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Glanders, caused by *Burkholderia mallei*, is a significant disease for humans due to the serious nature of the infection. It is recognized that *B. mallei* is an organism with tremendous infectivity that poses a significant hazard to humans exposed to aerosols containing this organism. Our knowledge of the pathogenesis of disease due to *B. mallei* is lacking. At present, no effective vaccines are available against this organism, and information on the treatment of this organism with antibiotic therapy is also not available. The basic studies that we are performing on the pathogenesis of disease due to *B. mallei* are acutely needed, and the information gained from these studies will provide a knowledge base that is required to rationally design new modes of therapy directed against this organism. The long-term objective of our research is to define at a molecular level the pathogenesis of disease due to *B. mallei* and to develop immunoprotective vaccines against these organisms for use in humans.

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INTRODUCTION

Glanders, caused by *Burkholderia mallei*, is a significant disease for humans due to the serious nature of the infection. It is recognized that *B. mallei* is an organism with tremendous infectivity that poses a significant hazard to humans exposed to aerosols containing this organism. Our knowledge of the pathogenesis of disease due to *B. mallei* is lacking. At present, no effective vaccines are available against this organism, and information on the treatment of this organism with antibiotic therapy is also not available.

The basic studies that we are performing on the pathogenesis of disease due to *B. mallei* are acutely needed, and the information gained from these studies will provide a knowledge base that is required to rationally design new modes of therapy directed against this organism. The long-term objective of our research is to define at a molecular level the pathogenesis of disease due to *B. mallei* and to develop immunoprotective vaccines against these organisms for use in humans.

Since glanders is of military significance as a biological warfare agent, the development of an effective vaccine and treatments are of particular concern. Our understanding of the disease caused by *B. mallei* is minimal, and we must move forward with these studies in order to develop new and effective vaccines and/or therapies against this organism. There is considerable dual use potential, since this disease is important in various areas of the world. Development of vaccines and treatments can, therefore, provide important items to assist the World Health Organization and to assist signatories of the Biological and Toxin Weapons Convention under Article X of the Convention.

BODY

***Burkholderia mallei* Challenge of Horses.**

We have initiated studies on experimental glanders in horses at the National Centre for Foreign Animal Diseases in Winnipeg, Manitoba. These studies were performed under the auspices of an Animal Use Document approved by CFIA and Health Canada. The studies were performed on miniature horses, approximately 120 Kg in weight. To initiate the infection, a 14 gauge needle was inserted through the chricothyroid cartilage, and an 8'' 16 g catheter was fed into the trachea to the bifurcation of the main bronchi and 4 ml *B. mallei* ATCC 23344 or *B. mallei* DD 3008 administered. Animals were monitored for 7 days.

Following are the necropsy reports on the seven horses studied to date:

Necropsy Report-Horse #1
***B. mallei* ATCC 23344**

The body submitted for necropsy on 6 July 2002 at approximately 0930 hrs is that of an adult approximately six-year-old approximately 120 kg intact male miniature horse, identified as No. 1, in good nutritional condition and in an excellent state of preservation. The post mortem interval is 30 minutes. There is a bilateral gray-white mucopurulent nasal discharge. The eyes are lightly encrusted with dried gray-white mucoid material. The coat is well-groomed. There are fresh venapuncture sites in the left jugular furrow. In the mucosal lining of the nasal septum bilaterally, there are multiple raised slightly firm round to irregular-shaped red to gray nodular foci of approximately 2-5 mm in diameter. The mandibular lymph node is mildly enlarged and slightly reddened. Within the proximal trachea between the first and second tracheal rings, just caudal to the larynx, is a reddened partially-healed approximately 2-3 mm diameter mucosal defect (interpreted as the percutaneous intratracheal inoculation site.) Upon incising the diaphragm, there is an inrush of air. The left apical lung lobe is reddened, non collapsed and slightly firm. Other lung lobes are collapsed and pink-colored. The mediastinal lymph nodes are minimally enlarged and reddened. There is marked splenomegaly. In the squamous (nonglandular) portion of the stomach, there are multiple attached dipterous larvae (bots). The stomach is nearly empty of ingesta. The small and large intestines are well-filled with moist digesta. The urinary bladder is moderately distended with clear pale yellow urine. Other organs are unremarkable.

GDx:

Body as a whole: Good nutritional condition and adequate hydration

Nasal septum: Acute rhinitis
Mandibular and mediastinal lymph nodes: Mild congestion or lymphadenopathy
Left apical lung lobe: Congestion or acute pneumonia
Spleen: Marked congestion ("barbiturate spleen")
Stomach: Infestation with *Gastrophilus intestinalis*, presumptive
Samples collected for bacteriology:
Lung, spleen, liver, kidney, nasal swabs, nasal septal mucosa and cartilage, mandibular lymph node, mediastinal lymph node, tracheal ring, urine.
Samples collected for nucleic acids:
Lung, spleen, liver
Samples collected for histopathology:
Palantine tonsil, muzzle, nostril, nasal septal mucosa, mandibular lymph node, thyroids, mediastinal lymph node, lung, heart, aorta, distal esophagus, tracheal ring, thymus, salivary gland, liver, spleen, adrenals, pancreas, mesenteric lymph node, stomach, duodenum, jejunum, ileum, cecum, dorsal colon, small colon, urinary bladder, testicle, brain, pituitary.
Comment: The lesion in the nasal septum is compatible with an acute upper respiratory tract infection, presumably induced experimentally. The lung lesion may also be a manifestation of experimental infection. The gastric endoparasitism is an incidental finding.

Reported by:

Catherine L. Wilhelmsen
DVM, PhD, Diplomate ACVP
Prosector

Necropsy Report Horse #2
***B. mallei* ATCC 23344**

The body submitted for necropsy on 6 July 2002 at approximately 1315 hrs is that of an adult approximately six-year-old approximately 110 kg intact male miniature horse, identified as No. 2, in good nutritional condition and in a very good state of preservation. The post mortem interval is nearly four hours. The coat is well-groomed. The body is in rigor mortis. There is bloody material in the left nostril. The eyes are thinly encrusted with dried yellow-gray mucoid material. There are fresh venapuncture sites in the left jugular furrow. In the mucosal lining of the left nasal septum, there are multiple ulcerated and deeply reddened areas. The mucosal lining of the right nasal septum is intact and mildly reddened. The mandibular lymph node is moderately enlarged, reddened, wet and bulging on cut surface. Within the caudal larynx, there is a faint mucosal defect (possibly the site of the percutaneous inoculation site.) The trachea is filled with blood-tinged froth. Upon incising the diaphragm, there is an inrush of air. All lung lobes are diffusely wet, heavy, non collapsed, dark tan-pink, with rib impressions and having scattered irregular linear, often chevron-shaped, red streaks or stripes. The cervical and mediastinal lymph nodes are moderately enlarged, reddened and bulging on cut surface. There is marked splenomegaly. In the squamous (nonglandular) portion of

the stomach, there are multiple attached dipterous larvae (bots). The stomach is nearly empty of ingesta. The small and large intestines are well-filled with moist digesta. The urinary bladder is mildly distended with clear medium-yellow urine. Other organs are unremarkable.

GDx:

Body as a whole: Good nutritional condition and fair state of hydration
Left nostril: Unilateral epistaxis
Left nasal septum: Unilateral ulcerative and hemorrhagic rhinitis
Mandibular, cervical and mediastinal lymph nodes: Moderate congestion or lymphadenopathy
All lung lobes: Severe diffuse pulmonary edema, with scattered linear hemorrhages or foci of congestion or pneumonia
Spleen: Marked congestion ("barbiturate spleen")
Stomach: Infestation with *Gastrophilus intestinalis*, presumptive

Samples collected for bacteriology:

Lung, spleen, liver, kidney, nasal swabs, nasal septal mucosa and cartilage, mandibular lymph node, cervical lymph node, mediastinal lymph node, laryngotracheal cartilage, urine.

Samples collected for nucleic acids:

Lung, spleen, liver

Samples collected for histopathology:

Muzzle, nostril, nasal septal mucosa, mandibular lymph node, thyroids, mediastinal lymph node, lung, heart, aorta, distal esophagus, tracheal ring, thymus, salivary gland, liver, spleen, adrenals, pancreas, mesenteric lymph node, stomach, duodenum, jejunum, ileum, cecum, dorsal colon, small colon, urinary bladder, testicle, brain, pituitary.

Comment: The ulcerative lesion in the left nasal septum is the likely source of the epistaxis. The lesion in the left nasal septum of this horse closely resembles the acute lesion depicted in colored plate XXXIX of an early reference. Epistaxis, without a history of previous work or other apparent cause, was reported in this same reference to be one of the frequent concomitant findings in glanders. The lung lesion observed in this horse may be a manifestation of experimental infection. The nature of the reddened streaks in the lung lobes awaits microscopic evaluation, but small V-shaped spots of pneumonia were reported in an early gross description of acute glanders. The severe pulmonary edema is interpreted to be a life-threatening lesion. This horse's life expectancy, had he not been euthanized, is judged likely to have been less than 12 hours. The gastric endoparasitism is an incidental finding.

Selected reference:

Huidekoper, RS, General diseases, In: Special Report on Diseases of the Horse, U.S. Department of Agriculture, Bureau of Animal Industry, Washington DC: Government Printing Office, 1907.

Reported by:

Catherine L. Wilhelmsen
DVM, PhD, Diplomate ACVP

Prosector

Necropsy Report Horse #3
***B. mallei* DD 3008**

The body submitted for necropsy on 29 October 2002 at approximately 0930 hrs is that of an adult approximately 113 kg intact male miniature horse, identified as No. 3, in good nutritional condition and in an excellent state of preservation. The post mortem interval is approximately 20 minutes. The mucosa of the soft palate is slightly roughened. At the tip of the right cardiac lung lobe, there is a slightly firm dark red-gray focus (possible chronic focal pneumonia; interpreted as old background lesion). There is moderate splenomegaly. Both chains of inguinal lymph nodes are slight prominent, firm and gray. The entire gastrointestinal tract is filled with normal green ingesta. Other organs are unremarkable.

GDx:

Body as a whole: Good nutritional condition and normal hydration
Right cardiac lung lobe: Chronic, focal, mild, pneumonia, presumptive
Spleen: Moderate congestion ("barbiturate spleen")

Samples collected for bacteriology:

Salivary gland, mandibular lymph node, laryngeal mucosa, tracheal mucosa, mediastinal lymph node, left and right apical, cardiac and diaphragmatic lung lobes), spleen, left and right kidneys, liver, left and right nasal septal mucosa, and nasal and laryngotracheal swabs.

Samples collected for histopathology:

Salivary gland, mandibular lymph node, tongue, soft palate with tonsillar tissue, larynx, thyroid, trachea, esophagus, haired skin, mammary gland, prepuce, heart, mediastinal lymph node, left and right apical, cardiac and diaphragmatic lung lobes, spleen, adrenal gland, left and right kidneys, mesenteric lymph node, liver, urinary bladder, testicles, stomach, pylorus, duodenum, pancreas, jejunum, ileum, ileocecal junction, proximal colon, distal colon, sciatic nerve, skeletal muscle, femoral bone marrow, left and right nasal septal mucosa, eyes, brain and pituitary.

Comment: The focal lung lesion is likely an incidental finding unrelated to experimental treatment.

Reported by:

Catherine L. Wilhelmsen
DVM, PhD, Diplomate ACVP
Prosector

Necropsy Report Horse #4
***B. mallei* ATCC 23344**

The body submitted for necropsy on 30 October 2002 at approximately 0930 hrs is that of an adult approximately 115 kg intact male miniature horse, identified as No. 4, in good nutritional condition and in an excellent state of preservation. The post mortem

interval is 15 minutes. The coat is fairly well-groomed. There is a fresh jugular venapuncture site. There is extensive ventral subcutaneous edema. On the ventrolateral surface of the tongue, there is a focal ulcer. There is bilateral mucopurulent ocular discharge and bilateral hemorrhagic and mucopurulent nasal discharge. The mucosal lining of the nasal septum bilaterally is ulcerated, with deep red (hemorrhagic) ulcer bases. Scant intact mucosa still lines the nasal septum. Remaining mucosal epithelium is roughened and lacks a glistening surface. The mandibular lymph node is moderately enlarged 2 to 3 times normal size, and is red-tan. The mucosa covering the epiglottis and the larynx has multiple coalescing ulcers, with yellow-gray to green-gray bases. The trachea is filled with pink-colored foam. The cranial tracheal mucosa is covered with numerous coalescing round to oval 0.5-1.0 cm diameter shallow ulcers having yellow-gray to green-gray bases and raised gray margins. The trachea for most of its length is flattened dorsoventrally, with a widened ventral ligament. The mediastinal lymph nodes are mildly enlarged and reddened (congested). Lung lobes diffusely are not collapsed and are slightly wet and heavy (diffuse pulmonary edema). Both cardiac lung lobes have locally extensively areas that are collapsed, deep red and slightly firm (atelctasis vs. acute pneumonia?). There is slight splenomegaly. The stomach is empty. The pancreas is slightly reddened. The small intestine contains scant fluid green digesta. The cecum and proximal colon are distended with fluid green contents. There are soft, moist feces in the distal colon. The urinary bladder contains a small volume of cloudy yellow urine. The cerebral gyri are slightly swollen, bulging and moist. Other organs are unremarkable.
GDx:
Body as a whole: Good nutritional condition and adequate hydration
Nasal mucosa: Acute necrotic hemorrhagic rhinitis
Larynx and trachea: Acute ulcerative laryngotracheitis
Trachea: Segmental partial collapse
Mandibular lymph node: Mild congestion
Lung lobes: Diffuse pulmonary edema with locally extensive atelectasis or acute pneumonia
Spleen: Mild congestion ("barbiturate spleen")
Brain: Possible cerebral edema
Samples collected for bacteriology:
Salivary gland, mandibular lymph node, laryngeal mucosa, tracheal mucosa, mediastinal lymph node, left and right apical, cardiac and diaphragmatic lung lobes), spleen, left and right kidneys, liver, left and right nasal septal mucosa, and nasal and laryngotracheal swabs.
Samples collected for histopathology:
Salivary gland, mandibular lymph node, tongue, soft palate with tonsillar tissue, larynx, thyroid, trachea, esophagus, haired skin, prepuce, heart, mediastinal lymph node, left and right apical, cardiac and diaphragmatic lung lobes, spleen, adrenal gland, left and right kidneys, mesenteric lymph node, liver, urinary bladder, testicles, stomach, pylorus, duodenum, pancreas, jejunum, ileum, ileocecal junction, proximal colon, distal colon, sciatic nerve, skeletal muscle, femoral bone marrow, left and right nasal septal mucosa, eyes, brain and pituitary.
Comment: The lesions in the nasal septum, larynx and trachea are compatible with an acute upper respiratory tract infection, presumably induced experimentally. The lung

lesion may also be a manifestation of experimental infection. The tracheal collapse may be a pre-existing abnormality.

Reported by:

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Prosector

Necropsy Report Horse #5
***B. mallei* ATCC 23344**

The body submitted for necropsy on 28 October 2002 at approximately 1400 hrs is that of an adult approximately 136 kg nongravid female miniature horse, identified as No. 5, in good nutritional condition and in a very good state of preservation. The post mortem interval is approximately 4 hours. There is copious bilateral mucopurulent nasal discharge. The coat is fairly well-groomed. There is a fresh jugular venapuncture site. The mucosal lining of the nasal septum bilaterally is markedly thickened (edematous). The nasal septal mucosal surface bilaterally is covered with erosions and shallow ulcers. The mandibular lymph node is enlarged four times normal size and is red-gray. The lymph nodes of the cervical chain are enlarged three times normal size and are red-gray. The mucosal lining of the soft palate and epiglottis has a cobblestone appearance (interpreted as mild lymphoid hyperplasia). A plug of yellow-tan mucoid material fills the larynx. After removing the pluck but before opening the larynx and trachea, yellow mucoid material exudes from the laryngeal opening. The trachea is filled with white froth and mucoid material. Upon opening the larynx, the laryngeal mucosa bilaterally is noted to have multiple erosions and shallow ulcers. The cranial tracheal mucosa is covered with multiple round to oval 0.5-1.0 cm diameter shallow ulcers having raised gray margins. When the lungs are elevated, a stream of straw-colored fluid runs from the lungs into the opened trachea. The cranioventral portions of both apical and cardiac lung lobes are consolidated, with firm knotty areas mottled red, yellow and gray-tan. Other lung lobes are not collapsed, wet and heavy (diffuse pulmonary edema). The right diaphragmatic lung lobe is pink-gray and the left diaphragmatic (downside) lung lobe is reddened (hypostatic congestion). The mediastinal lymph nodes are gray-colored and not enlarged. There is marked splenomegaly. The entire gastrointestinal tract is distended with bright green fluid contents. There are soft green feces in the distal colon. The urinary bladder is mildly distended with cloudy yellow urine. Other organs are unremarkable.

GDx:
Body as a whole: Good nutritional condition and adequate hydration
Upper respiratory tract: Acute erosive and ulcerative rhinitis and laryngotracheitis
Mandibular and cervical lymph nodes: Mild congestion or lymphadenopathy
Lung lobes: Acute locally extensive bronchopneumonia and diffuse pulmonary edema, presumptive
Spleen: Marked congestion ("barbiturate spleen")
Samples collected for bacteriology:

Salivary gland, mandibular lymph node, laryngeal mucosa, tracheal mucosa, mediastinal lymph node, left and right apical, cardiac and diaphragmatic lung lobes), spleen, left and right kidneys, liver, left and right nasal septal mucosa, and nasal and laryngotracheal swabs.

Samples collected for histopathology:

Salivary gland, mandibular lymph node, tongue, soft palate with tonsillar tissue, larynx, thyroid, trachea, esophagus, haired skin, mammary gland, heart, mediastinal lymph node, left and right apical, cardiac and diaphragmatic lung lobes, spleen, adrenal gland, left and right kidneys, mesenteric lymph node, liver, urinary bladder, ovaries, uterus, stomach, pylorus, duodenum, pancreas, jejunum, ileum, ileocecal junction, proximal colon, distal colon, sciatic nerve, skeletal muscle, femoral bone marrow, left and right nasal septal mucosa, eyes, brain and pituitary.

Comment: The lesions in the nasal septum, larynx and trachea are compatible with an acute upper respiratory tract infection, presumably induced experimentally. The lung lesion is likely a manifestation of experimental infection.

Reported by:

Catherine L. Wilhelmsen
DVM, PhD, Diplomate ACVP
Prosector

Necropsy Report Horse #6
***B. mallei* ATCC 23344**

The body submitted for necropsy on 28 October 2002 at approximately 1000 hrs is that of an adult approximately 136 kg nongravid female miniature horse, identified as No. 6, in good nutritional condition and in an excellent state of preservation. The post mortem interval is 30 minutes. There is copious bilateral mucopurulent nasal discharge. The coat is fairly well-groomed. There is a fresh jugular venapuncture site. The mucosal lining of the nasal septum bilaterally is markedly thickened (edematous). The nasal septal mucosal surface is bilaterally roughened, with erosions and shallow ulcers overlain with loosely adherent yellow-gray mucoid or necrotic material. The mucosal lining of the epiglottis has a cobblestone appearance (interpreted as mild lymphoid hyperplasia). The mucosa of the larynx has multiple erosions and ulcers, covered by a tightly adherent (diphtheritic) membrane. The trachea is filled with white froth and contains clumps of mucopurulent material. The mucosa lining the cranial trachea is covered with numerous round to oval 0.5-1.0 cm diameter shallow ulcers having raised gray margins. Within the cranial trachea at the level of the first and second tracheal rings, just left of the tracheal ligament, there is an oval 1 X 2 cm wide mucosal defect (deep ulcer or draining abscess). The mandibular lymph node is enlarged four times normal size, is tan-gray and wet on cut surface. The right prescapular lymph node is enlarged three times normal size. The mediastinal lymph nodes are gray-colored and are not enlarged. The cranioventral portions of both apical and cardiac lung lobes are locally mildly firm and reddened. Other lung lobes are expanded, slightly wet and heavy (diffuse pulmonary edema). The left lungs lobes are mildly reddened (hypostatic congestion). There is slight splenomegaly. The entire gastrointestinal tract is well filled with moist bright green

digesta. There are soft, formed feces in the distal colon. The urinary bladder contains a small volume of cloudy yellow urine. Other organs are unremarkable.

GDx:

Body as a whole: Good nutritional condition and adequate hydration
Upper respiratory tract: Acute necrotic rhinitis and laryngotracheitis
Mandibular and prescapular lymph nodes: Mild lymphadenopathy
Lung lobes: Acute locally extensive pneumonia and diffuse pulmonary edema, presumptive
Spleen: Mild congestion ("barbiturate spleen")

Samples collected for bacteriology:

Salivary gland, mandibular lymph node, laryngeal mucosa, tracheal mucosa, mediastinal lymph node, left and right apical, cardiac and diaphragmatic lung lobes), spleen, left and right kidneys, liver, left and right nasal septal mucosa, and nasal and laryngotracheal swabs.

Samples collected for histopathology:

Salivary gland, mandibular lymph node, prescapular lymph node, popiteal lymph node, tongue, soft palate with tonsillar tissue, larynx, thyroid, trachea, esophagus, haired skin, mammary gland, heart, mediastinal lymph node, left and right apical, cardiac and diaphragmatic lung lobes, spleen, adrenal gland, left and right kidneys, mesenteric lymph node, liver, urinary bladder, ovaries, uterus, stomach, pylorus, duodenum, pancreas, jejunum, ileum, ileocecal junction, proximal colon, distal colon, sciatic nerve, skeletal muscle, femoral bone marrow, left and right nasal septal mucosa, eyes, brain and pituitary.

Comment: The lesions in the nasal septum, larynx and trachea are compatible with an acute upper respiratory tract infection, presumably induced experimentally. The lung lesion may also be a manifestation of experimental infection.

Reported by:

Catherine L. Wilhelmsen
DVM, PhD, Diplomate ACVP
Prosector

Necropsy Report Horse #7
***B. mallei* ATCC 23344**

The body submitted for necropsy on 29 October 2002 at approximately 1230 hrs is that of an adult approximately 120 kg intact male miniature horse, identified as No. 7, in a state of nutritional over condition and in an excellent state of preservation. The post mortem interval is approximately 15 minutes. The ventral neck region is extremely thickened and indurated. Both jugular furrows, obscured by the neck swelling, are not discernable. The skin of the ventral neck region is tightly adherent to firm, fibrotic subcutaneous tissue. The subcutis is approximately 2 cm thick. Midway between the throat and the thoracic inlet, an oval 2 X 2 X 6 cm pocket of yellow purulent material surrounded by a fibrous capsule, is embedded in the thickened subcutis. The trachea, just dorsal to this pocket, is moderately flattened dorsoventally. The narrowed tracheal lumen is oval in cross-section. The trachea is filled with white froth. The lungs are mildly

expanded and wet. There is excessive fat in all body fat depots. There is moderate splenomegaly. Mediastinal and most peripheral lymph nodes are difficult to localize in the excessive body fat. Mesenteric lymph nodes are readily identified as they are slightly enlarged, prominent, and reddened. The stomach is nearly empty. Within the stomach, scant fresh red (undigested) blood coats the mucosa. There are several irregular round to oval gastric mucosal erosions or shallow ulcers with overlying adherent green material (possibly either ingesta or a diphtheritic membrane). The entire intestinal tract is filled with normal green ingesta. Other organs are unremarkable.

GDx:

Body as a whole: Mild obesity and normal hydration
Ventral cervical region: Chronic-active locally extensive cellulitis with fibrosis and focal chronic abscess
Trachea: Segmental partial collapse
Lung lobes: Mild diffuse pulmonary edema
Stomach: Acute erosive to ulcerative gastritis
Spleen: Moderate congestion ("barbiturate spleen")
Mesenteric lymph nodes: Mild congestion

Samples collected for bacteriology:

Salivary gland, mandibular lymph node, laryngeal mucosa, tracheal mucosa, mediastinal lymph node, left and right apical, cardiac and diaphragmatic lung lobes), spleen, left and right kidneys, liver, left and right nasal septal mucosa, nasal and laryngotracheal swabs, cervical lymph node and subcutaneous abscess.

Samples collected for histopathology:

Salivary gland, mandibular lymph node, tongue, soft palate with tonsillar tissue, larynx, thyroid, trachea, esophagus, haired skin, prepuce, heart, mediastinal lymph node, left and right apical, cardiac and diaphragmatic lung lobes, spleen, adrenal gland, left and right kidneys, mesenteric lymph node, liver, urinary bladder, testicles, stomach, pylorus, duodenum, pancreas, jejunum, ileum, ileocecal junction, proximal colon, distal colon, sciatic nerve, skeletal muscle, femoral bone marrow, left and right nasal septal mucosa, eyes, brain, pituitary and subcutaneous abscess.

Comment: The chronicity of the cellulitis and abscessation suggests that this condition was acquired prior to the horse's arrival at NCFAD. It is possible, but not proven, that the tracheal partial collapse may have been the result of compression exerted on the trachea by the developing inflammatory process. (An alternative explanation is that the tracheal partial collapse may have be a co-incidental finding unrelated to the inflammatory lesion.) The mild gastritis may have been induced by a host stress response, since there is no known history of recent administration of nonsteroidal anti-inflammatory agents, another common cause of erosive gastritis in horses.

Reported by:

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Prosector

It is apparent that we have developed a model of experimental glanders in horses. Further, we have demonstrated that a capsule minus mutant of *B. mallei* is avirulent in the horse. To date, we have drawn the following conclusions.

- The pathogenesis of disease due to *B. pseudomallei* and to *B. mallei* is complex and multifactorial.
- Vaccine development for melioidosis and for glanders is proceeding.
- Capsule (PS) is a major virulence determinant of *B. mallei*.
- Testing of rETA-PS conjugate vaccine against glanders in the horse model has the potential to allow us to draw parallels to the closely related disease, melioidosis, in humans.
- Numerous strategies for vaccine development exist including conjugate, DNA, attenuated and heterologous vaccines, all of which have demonstrated potential for further development.
- We must continue to study the underlying mechanisms of *B. pseudomallei* and *B. mallei* pathogenesis to identify new potential targets for immunoprophylaxis.

The next steps are to perform dose-response studies in additional animals to determine the minimum numbers of organisms required to initiate an infection. Following these dose-response studies, we will initiate challenge studies of immunized animals with 10x and 100x the minimum dose of *B. mallei* required to initiate infection in the horse.

Type III Secretion System Studies

Type III secretion systems (TTSS's) of *B. pseudomallei* are being studied with an aim at defining how these systems contribute to pathogenicity. Our first line of approach is to mutagenize each of the three TTSS clusters identified from the Sanger chromosomal sequence, and subsequently assay for phenotypes associated with each TTSS cluster. One of the TTSS machinery subunits present in each of the three clusters is the SctU subunit, and it has therefore become the target of this approach. A recent publication also deleted SctU from the *Salmonella*-like *B. pseudomallei* TTSS cluster (TTSS3), and found that the TTSS3 cluster is required for persistence and replication of *B. pseudomallei* within a macrophage cell line (Stephens, *et al.*). I have generated a $sctU_{Bp3}$ mutant (*sctU* gene from *B. pseudomallei* TTSS3) and found that this mutant resulted in no significant attenuation of virulence in a hamster model of infection. We reason that this result is not in conflict with the result by Stephens *et al.* because two additional copies of *sctU* exist in the *B. pseudomallei* chromosome ($sctU_{Bp1}$ and $sctU_{Bp2}$) that may complement deletion of $sctU_{Bp3}$ over the longer course of an animal infection compared to a short course tissue culture model of infection. I am therefore generating a $sctU_{Bp123}$ triple mutant (completion due in Feb 2003) that we anticipate will be attenuated in animal infection models.

Given the importance of the *Salmonella*-like TTSS cluster (TTSS3) in the survival of *B. pseudomallei* in eukaryotic cells, I am also performing mutagenesis of TTSS3-delivered effector molecules. These putative effectors include BopA (PMN transmigration), BopE (invasion), BipB (apoptosis inducer), and BipC (actin nucleation). In addition, three orf's are predicted to generate the putative effectors BapA, BapB, and BapC – proteins with no known homologues. Mutants for *bopA*, *bopE*, and *bapC* have already been cloned and are currently being inserted into the chromosome for analysis. Antibodies are also being generated against these proteins. Mutants of *bapA* and *bapB* are still being cloned. BipB and BipC are not currently being analysed due to their predicted intrinsic necessity in effector molecule delivery through pore formation, making it very difficult to analyse functions inside host cells beyond pore formation (effector delivery). A mutant is also being generated of the major structural subunit of a type IV pilus, PilS. The pilus gene cluster is located within the TTSS2 cluster.

Subsequent to the generation of an array of mutants, they will be examined by both animal infections and by infections of cultured cell lines. The use of cultured cell lines will assist in pinpointing specific roles for each of the putative effector molecules, and it is anticipated that novel *B. pseudomallei* phenotypes will be identified through this analysis.

Through a student project, we will also be investigating the role of three proteases whose genes are located adjacent to the TTSS3 cluster. These proteases are homologues of thermolysin, serine carboxypeptidase, and a serine peptidase. The genes will be cloned into expression vectors, and their putative proteolytic functions will be demonstrated through simple assays.

Random Promoter Library Construction and Analysis

Certain mechanisms of *B. mallei* pathogenesis have been identified such as the production of capsule, but it is believed that more virulence determinants have yet to be identified. In order to better understand the pathogenesis of *B. mallei*, a promoter library will be constructed. The library will use random genomic fragments from *B. mallei* as the promoter for the *lux* reporter system. Expression of the *luxCDABE* operon results in luminescence which is easily measured. The promoter library will be grown under conditions thought to mimic *in vivo* conditions, which should allow the identification of genes expressed *in vivo*. Some of these genes will function in the pathogenesis of *B. mallei*.

The *B. mallei* promoter library will be screened under conditions thought to mimic the conditions of infection. Initial screens will be done using varying concentrations of iron magnesium and manganese. Low levels of iron have been shown to induce the production of virulence factors in a large number of bacteria (Ratledge and Dover 2000). Recent studies have shown that manganese plays a role in the virulence of some species of bacteria (Boyer, Bergevin et al. 2002). It is thought that manganese can compensate for low levels of iron in some bacteria. Enzymes such as manganese superoxide dismutase, function to protect the bacteria from reactive oxygen species, which are produced *in vivo* by host cells in response to bacterial infection (Jakubovics and Jenkinson 2001). Low levels of magnesium have been shown to induce the production of virulence factors in some bacteria (Groisman 2001; Johnson, Newcombe et al. 2001). Genes that are expressed at higher levels under high manganese, low iron or low magnesium conditions may be involved in the pathogenesis of *B. mallei*.

The promoter library will also be screened for altered luminescence when the bacteria are grown in the presence of eukaryotic cells and sera. During infection *B. mallei* comes into contact with host cells. Contact with host surface structures or secreted products may induce the expression of virulence determinants in *B. mallei*. These virulence determinants may be identified by screening the promoter library for altered gene expression in presence of eukaryotic cells. *B. mallei* can be isolated from the blood of infected individuals, so it is likely that *B. mallei* has mechanisms to prevent killing by complement. By exposing the *B. mallei* promoter library to sera, we should identify genes which prevent killing by complement.

The promoters which induce a high level of *lux* expression under *in vivo*-like growth conditions will have the promoter sequenced. The sequences will be used to identify the genes which are controlled by the promoter. Selected genes will be investigated further to determine if they have a role in the virulence *B. mallei*. The analysis of the gene sequences will determine the type of investigation done. Genes with homology to known virulence factors of other bacteria could be disrupted in the chromosome of the *B. mallei* and the resulting strain tested for altered virulence. Proteins which are secreted from the bacteria could be over expressed and tested for function as toxins. Surface proteins could be tested to determine if host immune responses to these proteins are protective during

infection. The exact nature of the investigations done will be dependent on the types of genes selected.

The results of the screen of the *B. mallei* promoter library will be compared to the results of other promoter library screens. *B. mallei* is closely related to *B. pseudomallei* and *B. thailandensis*. A *B. pseudomallei* promoter library has been made by Jessmi Ling and is being screened. *B. thailandensis* is a non-pathogenic species found in soil. A *lux* promoter library of the *B. thailandensis* strain E264 will also be made and screened. The results of the screens of the three libraries will be compared. Genes that are expressed under *in vivo* conditions in the pathogen strains but not in *B. thailandensis* may have a role in virulence. Conversely, genes which are expressed in the pathogen strains as well as *B. thailandensis* are less likely to have a role in the virulence of the pathogens. Differences in the expression patterns of the pathogen strains may explain the differences in the diseases caused by the two species of bacteria.

The strain of *B. mallei* which is being used is the type strain ATCC 23344. This strain has been used with a number of disease models and methods for genetic manipulation of it have been developed. To construct the promoter library, genomic DNA from ATCC 23344 will be cloned into pMS402. The plasmid pMS402 can replicate in both *E. coli* and *Burkholderia* species, and codes for resistance to both kanamycin and trimethaprim. Partial digestion of the genomic DNA with *Sau*3A1 will be used produce DNA fragments of 850 to 1600 bp, which will be cloned into a *Bam*H1 site in upstream from the *luxCDABE* operon. The transfer of the genomic library into *B. mallei* will be done by mating with *E. coli*. The plasmids can be transferred by mating *E. coli* S-17 Lambda pir containing the pMS402 library with ATCC 23344, or by tri-parental mating with *E. coli* HB101 pRK20B, *E. coli* Electromax DH10b cells (Gibco Life Sciences) containing the pMS402 library and ATCC 23344. The genomic library will be screened for luminescence to identify clones containing promoters upstream of the *luxCDABE* operon. The promoter library will be composed of the clones that show luminescence when grown in both rich and minimal media.

This general procedure will also be used to generate a promoter library of *B. thailandensis* strain E264. Strain E264 is the type strain for the species and has been confirmed to be avirulent in the Syrian golden hamsters (Brett, DeShazer et al. 1997).

The construction of both promoter libraries has started. The genomic DNA fragments have been cloned in to the pMS402 plasmid. Transfer of the plasmids into *B. mallei* ATCC 23344 and *B. thailandensis* E264 is in progress. To represent the entire genome of *B. mallei* approximately 18000 clones will be checked required. Twenty thousand clones will be required to represent the *B. thailandensis* genome. Due to the size of the libraries required the screening of the initial clones will be done concurrently with the production of the rest of the libraries.

Studies on ß-lactamase in *B. thailandensis* and *B. pseudomallei*

Construction of ß-lactamase-lux fusions

In order to examine expression of ß-lactamase in *B. pseudomallei* we employed a *lux*-based suicide vector which allows inactivation of targeted genes (in this case ß-lactamase) and *lux*-dependent light production as a result of gene expression. The vector pGSV-*lux* was constructed by cloning the *lux* operon from pCS46 into the *Not*1 site of the suicide vector pGSV. An internal region of the *penA* gene was then amplified via PCR and cloned into pCR2.1 (Invitrogen). Once cloned into pCR2.1, the fragment was cleaved with *EcoR*1, isolated via agarose gel electrophoesis and cloned into the *EcoR*1 site of pGSV-*lux*. The pGSV-*lux-penA* construct was then transformed into *E.coli* Top10 cells , subsequently purified and transformed into the conjugative *E. coli* strain SM10 λ-*pir*. The plasmid pGSV-*lux-penA* was transferred to *B. thailandensis* and *B. pseudomallei* via overnight conjugation and single cross over events selected for by plating the mating mix onto LB agar plates containing 100 ug/ml polymyxinB and 25 ug/ml gentamicin. Transconjugants were tested for *lux*-mediated light production by transferring individual colonies to a 96 well plate containing LB –gentamicin broth and measuring light production after overnight incubation at 37° C in a Victor luminometer. One light producing *B. thailandensis* transconjugant , RM700, was selected and used for further studies. To determine that the lux operon was in the correct orientation within the penA structural gene PCR amplifications were performed in which a primer from the 5' end of the penA gene (primer 5'-bla) and a primer within the *lux* E gene (primer Zeo6) were used to generate a predicted ca. 900 bp PCR product. As this product could only be amplified if the lux genes had inserted in the same orientation as the *penA* gen gene, these results suggested that the lux operon had inserted in the correct orientation. The 900 bp PCR product was cloned into pCR2.1 and sequenced in both directions. The results from sequencing confirmed that the *lux* operon had inserted in the same orientation as the *penA* gene and that light production in strain RM700 was a reflection of *penA* gene expression.

ß-lactam MICs in *B. thailandensis* RM700

ß-lactam MICs were determined in *B. thailandensis* RM700 using E-tests. The results are shown below. Of the variety of ß-lactams tested only MICs to ampicillin, amoxicillin and ceftriaxone were markedly decreased as a result of the *penA* disruption whereas MICs to ceftazidime, pipericillin, oxacillin and imipenem were not, indicating that the latter group of antibiotics are likely poor substrates for the *penA* encoded ß-lactamase.

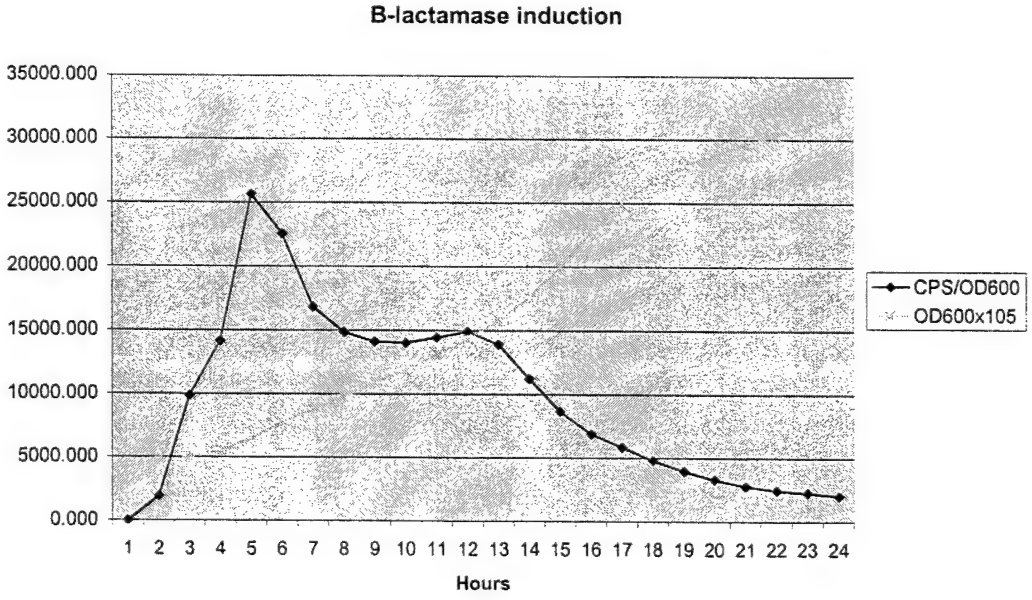
Table 1. ß-lactam MICs of *B. thailandensis* RM700

ß-lactam	Strain	
	RM700	DW503
Ampicillin	3	16
Pipercillin	1	3
Ceftazidime	0.75	1.0
Amoxicillin	4	96
Oxacillin	>256	>256
Imipenem	0.125	0.38
Ceftriaxone	1.0	>32

Expression of ß-lactamase in *B. thailandensis* RM700

The expression of ß-lactamase in *B. thailandensis* RM700 was examined by monitoring light production over a period of 24 hours. The pattern of expression was consistently similar between experiments and the results of a typical experiment are shown below. Strongest light emmision was observed in early exponential phase 3-4 hours after inoculation. Light emmision delined afterwards until about 12 hours, where a small increase in light production occurred followed by a steady drop. A similar pattern of ß-lactamase activity was observed in *B. thailandensis* DW503 using the colormetric substrate nitrocefin to measure enzyme activity.

Figure 1. ß-lactamase expression in *B. thailandensis* RM700

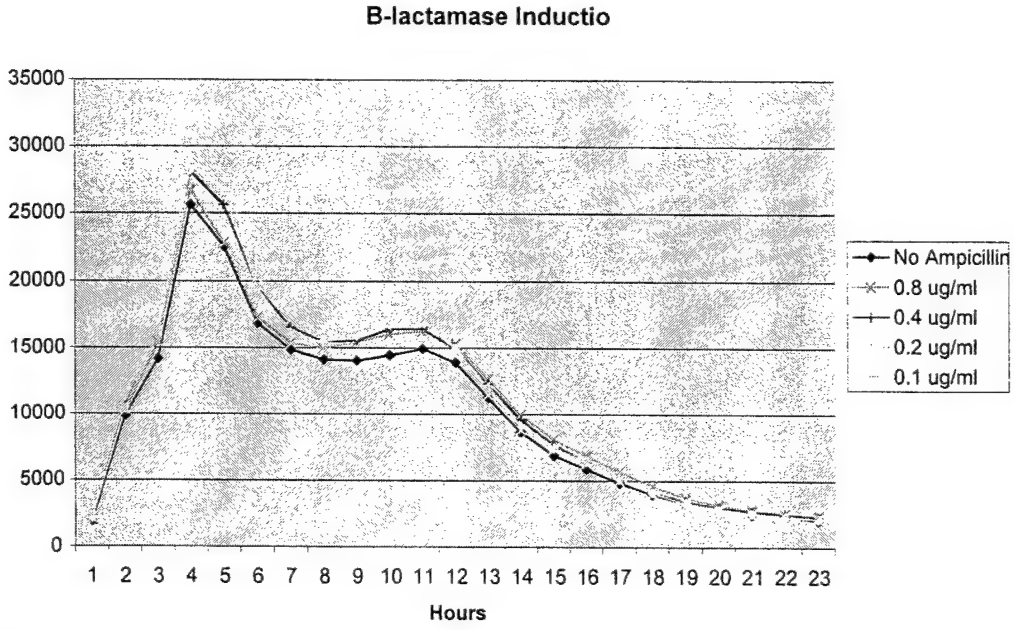


CPS/OD = counts per minute (light production) divided by optical density at 600 nm.

Induction of ß-lactamase in *B. thailandensis* RM700

Previous studies have reported that ß-lactamase is an inducible enzyme in *B. pseudomallei*. We attempted to induce ß-lactamase in *B. thailandensis* by growing the organism in sub-MIC levels of antibiotic. Enzyme expression was measured by monitoring light emission over a period of 24 hours. As seen in figure 2 (below) there was no apparent enzyme induction in the presence of various amounts of ampicillin. Similar experiments performed with imipenem, considered to be a "good" inducer of ß-lactamase in many gram negative bacteria, were also negative.

Figure 2. Induction of ß-lactamase in *B. thailandensis* RM700



Further studies

B. pseudomallei ß-lactamase

We have recently constructed equivalent mutants in *B. pseudomallei* 1026b and will soon examine the expression, induction and regulation of ß-lactamase in these strains

Complementation studies

Complementation experiments will be performed using PCR amplified *penA* cloned into the broad host range plasmid pBHR1 gene and conjugated into both *B. thailandensis* and *B. pseudomallei* to determine if expression profiles observed in *penA-lux* mutant strains are altered in the presence of functional enzyme. In addition, the promoter region of the

penA gene will be cloned into a *lux*-promoter vector to determine if similar results observed with the *penA* -lux fusions are obtained with an alternative reporter system.

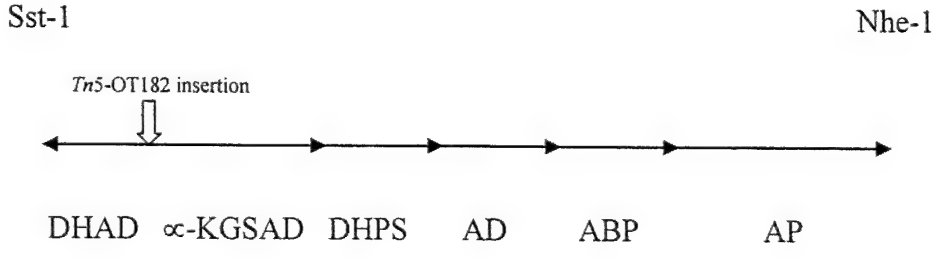
Arabinose metabolism in *B. thailandensis*

Arabinose utilization has historically been used to differentiate virulent v.s. avirulent *B. pseudomallei* strains. Ara+ strains are now known to be *B. thailandensis* wheras Ara- strains are *B. pseudomallei.* We had originally thought to examine arabinose metabolism genes in *B. thailandesis* and determine if there was any relationship to avirulence. One possibility for this type of relationship would be a situation where the arabinose metabolizing genes had integrated into virulence genes

To identify arabinose metabolism genes, *B. thailandensis* E264 was mutagenized with the transposon *Tn5*-OT182 and mutants unable to grow using arabinose as a sole carbon source were isolated. Mutants were screened further by testing for the ability to grow using glucose as a sole carbon source since mutants unable to grow on both arabinose and glucose would indicate a mutation which was likely involved in some aspect of carbohydrate metabolism not directly associated with arabinose.

We identified one transposon mutant which was unable to grow on arabinose media solely, and choose this strain for additional study. Approximately 6 kb of DNA flanking the transposon integration was cloned following *Nhe*-1 digestion of chromosomal DNA. The resulting flanking DNA clone, pNhe was sequenced, and the transposon integration found to be located in an open reading frame with strong homology to gene encoding the enzyme ∝–ketoglutarate semi-aldehyde dehydrogenase (∝-KGSAD). DNA flanking the opposite side of the transposon integration was cloned following *Sst*-1 digestion of chromosomal DNA however this clone, pSst ,extended only 200 bps beyond the transposon integration and has thus provided only limited information about other metabolic genes. Sequencing of flanking DNA revealed several open reading frames with homology to known arabinose metabolism genes. These are shown below.

Figure 3. Arrangement of arabinose metabolism genes in *B. thailandensis* E264.



(DHAD= Dihydroxy-acid dehydratase, ∝-KGSAD=∝–ketoglutarate semi-aldehyde dehydrogenase, DHPS = Dihyropicolinate synthase, AD= Arabinose dehydrogenase, BP = Arabinose binding protein, AP = Arabinose permease)

BLAST search homologies resulting from sequence information has allowed prediction of a likely metabolic pathway for arabinose in *B. thailadensis* based on known arabinose metabolic pathways in gram-negative bacteria. This pathway is shown below.

Figure 3. Putative arabinose metabolism pathway in B. thailandensis.

L-arabinose

⇓ (Outer membrane protein)

⇓ (arabinose periplasmic binding protein) √

⇓ (arabinose permease) √

⇓ (arabinose dehydrogenase) √

L-arabinolactone

⇓ (arabinolactonase)

L-arabinoate

⇓ (L- arabinoate dehydratase)

2-keto-3-deoxy-L-Arabonate

⇓ (2-keto-3-deoxy-arabinoate aldolase)

∝–ketoglutarate semialdehyde

⇓ (∝–ketoglutarate semi-aldehyde dehydrogenase) √

∝–ketoglutarate

(A √ located next to the enzyme indicates identification of a homolog in the sequence data obtained thus far.)

Further studies

We plan to continue identification of arabinose metabolic genes and to determine where these genes are located relative to adjacent genes in the *B. thailandensis* chromosome. Adjacent genes will be examined to determined if they have homologs in *B. pseudomallei* and *B. mallei*.

Microarray Studies

We have developed 35mer oligo-microarrays of 164 genes of *B. pseudomallei* and *B. mallei*, and the slides will be used to study gene expression in these two bacterial species in different growth conditions such as in different concentrations of magnesium and iron. In the near future, we use this approach to study gene expression of bacteria during infection of tissue culture cells and also in *in vivo* studies in animal models.

We have identified 3 goals for our microarray studies:

1) To develop DNA microarray technology as a high throughput tool to study gene expression in *B. pseudomallei* and *B. mallei*.

2) To study the roles of metals such as magnesium and iron in gene expression in these pathogens.

3) To utilize DNA microarray technology to study bacterial pathogenesis in tissue culture cells and in animal models.

DNA microarray is a high throughput technology used to study gene expression in various organisms based on the comparison of the mRNA populations from either different growth conditions or treatments. However, DNA microarray analysis in bacteria is not widely used since microarray slides are commercially available for only some bacterial species, such as *Escherichia coli* and *Pseudomonas aeruginosa*. Thus, the construction of whole genome microarrays for certain bacterial species may take time and money. However, our interests are mainly in the area of bacterial pathogenesis so that the construction of whole genome microarrays may not be necessary, and groups of genes hypothesized to be involved in bacterial pathogenesis only are included in the construction of the arrays.

Experimental procedures

Microarray technology is based on the comparison of mRNA populations from two or more conditions of bacterial growth in terms of types and amounts. Thus, the techniques in the microarray analyses include the preparation of the bacterial RNA in order to generate the fluorescence probes and the hybridization with the oligonucleotide targets

which are immobilized on the glass substrate. In this study, we started with the optimization of all conditions needed for bacterial total RNA preparation and for making the cDNA probes. The direction of the investigation is shown in figure 1, and details of the experiment are given as follows:

Bacterial strains and growth conditions:
Burkholderia pseudomallei strain 1026B and *B. mallei* ATCC 23344 were used in this study. The roles of magnesium and iron in gene expression of these bacterial strains were investigated. The minimal salt medium, M9 and TSBDC (tryptic soy broth treated with Chelex-100, dialysed and supplemented with 50 mM glutamate and 1% glycerol) were used. The culture treated with 20mM $MgSO_4$ and 200 □M $FeCl_3$ were set as the high magnesium and high iron conditions, respectively. The bacterial culture without adding those elements was set as the control of the low metal conditions. The bacterial cells were grown in 75 ml of the culture medium at 37 °C with a rotational shaking at 250 rpm, 8-10 hrs to reach the mid-late exponential phase or $1.2 - 1.4 \times 10^7$ cells/ml.

Total RNA isolation:
Total RNA was isolated from 70 mL of the broth culture by using a phenol-chloroform based technique. The RNA*Later* and RNA*wiz* reagent kits from Ambion Inc, USA were used in this process with a modification recommended by the manufacturer. The total RNA from this technique yielded 4-6 □g/□l. Figure 2 shows the electrophoresis profile of the total bacterial RNA produced from this technique.

cDNA generation and making the probe:
To generate the fluorescence-labeled cDNA, the total RNA was annealed to the random primers $pd(N)_6$ (Amersham Pharmacia, USA) and the cDNA was synthesized by a reverse transcription. The amino allyl modified dUTP (aa-dUTP) was randomly added onto the first stranded cDNA, which provided the conjugation sites for the binding with the fluorescence dyes containing either a HNS- or STP-ester leaving groups. Two fluorescence dyes, Cy3 and Cy5 were used in this labeling technique. FairPlay™ Microarray labeling kit (Stratagene, USA) and a protocol from the manufacturer with some modification were used in this study.

Hybridization and washing:
The hybridization was performed at 37 °C with 100% humidity for at least 18 hours. The washing step in this study was based on a standard protocol.

Scanning and data analysis:
The scanning of microarray slides is based on the detection of two different fluorescence signals from Cy3 and Cy5 at the wavelengths 550 and 650 nm, respectively. The fluorescence intensity of each spot of the microarray were recorded and normalized by using the QuantArray microarray analysis software (Packard Bioscience, USA). The comparison of the intensities provide the expression level between the control and the testing conditions. To determine the significance of the measurement, *t*-test and ANOVA (Analysis of Variance) are used in the analysis.

Design and making the 35mer oligo-microarray

35mer oligo-microarrays for *B. mallei* and *B. pseudomallei* have been made for testing 164 genes from the published sequences and from the computerized prediction from the genome sequences obtained from the Sanger Institute and TIGR. This microarray contains genes encoded for different functions in bacterial pathogenesis, such as the capsular biosynthesis genes, protease genes, antibiotic resistance genes and type III secretion system. While the another group of the genes are the maintenance genes, such as the genes for metabolic pathways, and genes encode for the bacterial structural components such as the flagellin gene, pilus genes and the O-antigen biosynthesis genes. The idea of this design is to use the maintenance genes which are conserved in both bacterial species to show the expression level differences in different growth conditions of the bacteria, while the pathogenesis and virulence genes are used for determining the conditions necessary for the gene expression during the bacterial infection either in the tissue culture cells or the animal models. We expect that the results from this microarray would provide significant information on gene expression and pathogenesis in these bacterial species.

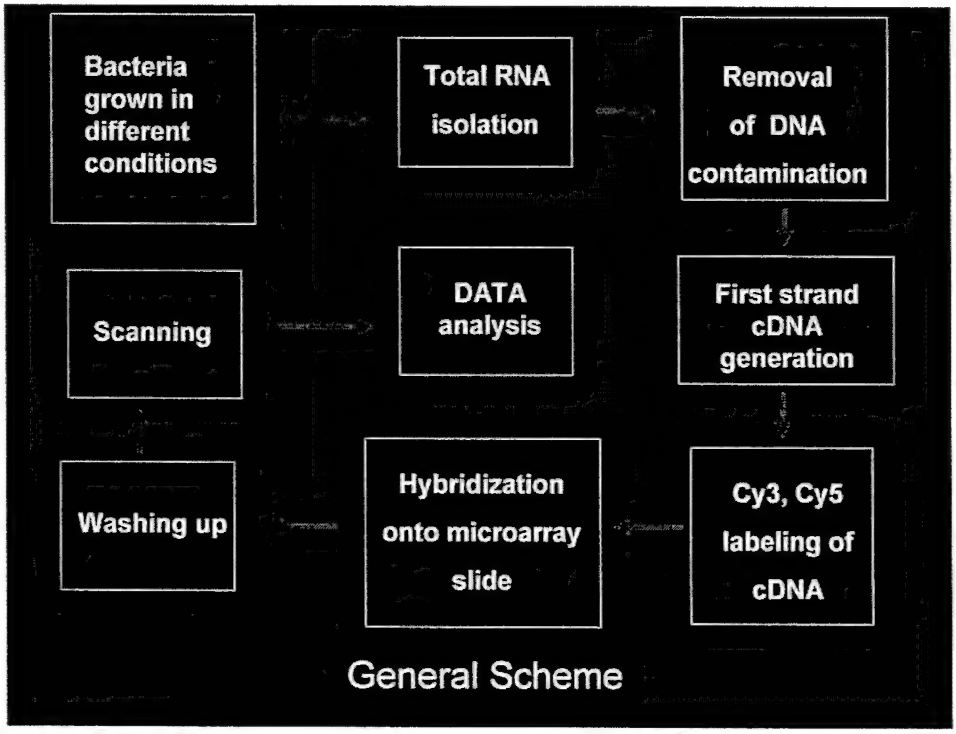
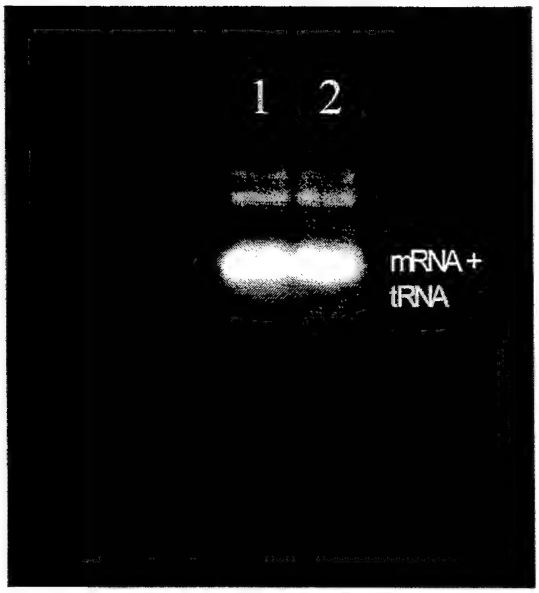


Figure1. General Research Scheme.



Total RNA from 75 mL of TSB-DC broth culture and purified with DNA-free ® (Ambion); Lane 1, grown w/o Mg; Lane 2, w/ Mg

Figure2. The electrophoresis profiles of total RNA isolated from *B. mallei* using the phenol-chloroform based extraction method.

Preliminary results

During the process of optimizing the 35mer oligo microarray to test the roles of iron in gene expression in both bacterial species, the results showed that most genes expressed equally in *B. mallei* in conditions of high and low concentrations, while the expression profile in *B. pseudomallei* showed about five fold higher expression in high iron concentrations. However, the results from this optimization step are not significantly confirmed, since there are several remaining steps ahead to determine the significance of this measurement.

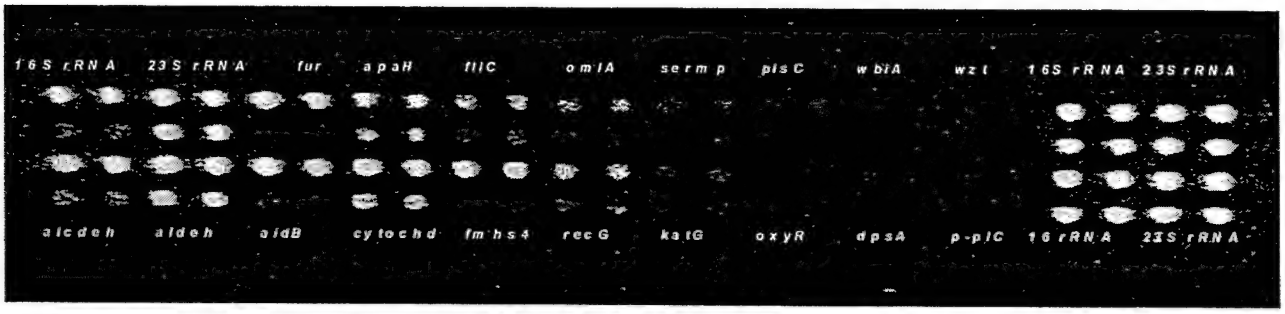


Figure3. An example of gene expression profiles in *B. mallei* using 35mer oligo-microarray of 20 tested genes; low iron (green) VS high iron (red), yellow = equal expression.

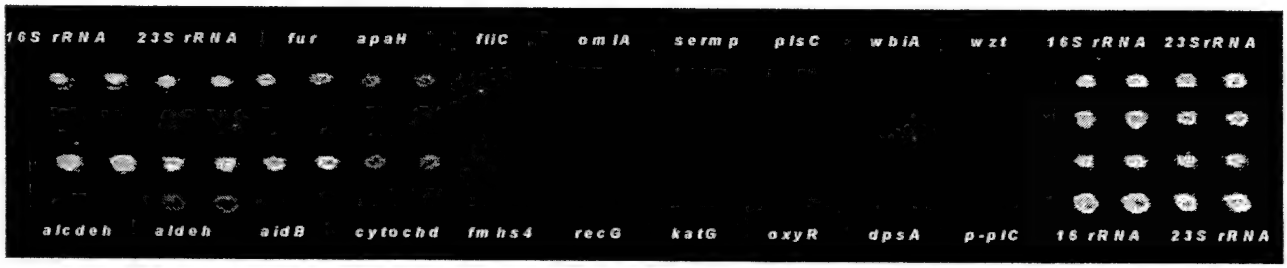


Figure4. An example of gene expression profiles in *B. pseudomallei* using 35mer oligo-microarray of 20 tested genes; low iron (green) VS high iron (red), yellow = equal expression.

Further studies

Within the first year term of the study, we would like to use the 35mer oligos to investigate the roles of metals such as iron and magnesium in gene expression of these two bacterial species. More gene sequences will be added to the recent 164 gene microarray if necessary. The development of microarray techniques to study macrophage cells during bacterial infection will start as soon as we obtain enough baseline data from the *in vitro* study in broth culture. Studies in animal modes will follow.

Key Research Accomplishments

- We have been able to confirm via the use of ^{1}H and ^{13}C NMR spectroscopy that the O-PS antigen expressed by *B. mallei* GB8 is an unbranched polymer of repeating disaccharide units having the structure –3)-β-D-glucopyranose-(1-3)-6-deoxy-α-L-talopyranose-(1- in which the L-6dTal*p* residues bear 2-*O*-methyl or 2-*O*-acetyl substitutions.
- Through the use of a polyclonal antiserum and a monoclonal antibody raised against *B. pseudomallei* O-PS antigens we have begun to assess the immunogenic similarities between *B. mallei* and *B. pseudomallei* O-PS molecules. These studies are important for determining whether or not a conjugate vaccine composed of only *B. pseudomallei* antigens would afford protection against *B. mallei* as well as *B. pseudomallei*. The initial results demonstrate that while the O-PS moieties expressed by the two species differ to some degree, the polyclonal antiserum is capable of reacting strongly with both.
- We have demonstrated that the presence or absence of O-acetyl groups on the O-PS moieties may have consequences when considering O-PS as a component of a vaccine that protects against both *B. mallei* and *B. pseudomallei.*
- We have completed a number of studies on antibiotic resistance in *B. mallei*. We have demonstrated that the selection of antibiotics that have proper targets is important. Furthermore, the selective pressure from antibiotic use has a strong influence for the emergence of the mutant enzymes. The physician may have to consider multiple drug regimens in the treatment of diseases caused by *Burkholderia spp*, in order to prevent the development of resistance.
- We have developed a model of experimental glanders in horses. Further, we have demonstrated that a capsule minus mutant of *B. mallei* is avirulent in the horse.
- The pathogenesis of disease due to *B. mallei* is complex and multifactorial. Vaccine development for glanders is proceeding. Capsule (PS) is a major virulence determinant of *B. mallei*. Testing of rETA-PS conjugate vaccine against glanders in the horse model has the potential to allow us to draw parallels to the closely related disease, melioidosis, in humans.
- Numerous strategies for vaccine development exist including conjugate, DNA, attenuated and heterologous vaccines, all of which have demonstrated potential for further development.
- We must continue to study the underlying mechanisms of *B. mallei* pathogenesis to identify new potential targets for immunoprophylaxis.

Reportable Outcomes

- We have developed a model of experimental glanders in horses. Further, we have demonstrated that a capsule minus mutant of *B. mallei* is avirulent in the horse.
- We have published a manuscript describing the molecular and physical characterization of *Burkholderia mallei* O-antigens. This manuscript is attached as Appendix 1 and has been published in the Journal of Bacteriology.
- We have published a manuscript describing a temperate phage from *B. thailandensis* specific for *Burkholderia mallei*. The manuscript is attached as Appendix 2 and has been published in the Journal of Bacteriology.
- We have published a manuscript describing a locus required for 2-0-acetylation of O-antigens expressed by *Burkholderia pseudomallei* and *B. thailandensis*. The manuscript is attached as Appendix 3 and will be published in FEMS Microbiology Letters.
- We have published a manuscript describing the characterization of class A β-lactamase mutations of *Burkholderia pseudomallei* that confer selective resistance against ceftazidime or clavulanic acid inhibition. The manuscript is attached as Appendix 4 and will be published in Antimicrobial Agents and Chemotherapy.
- Dr. Apichai Tuanyok is a postdoctoral fellow currently supported by this contract.
- We have applied and been approved for a renewal of a Canadian Institutes of Health Operating Grant based on work supported in part by this contract.

Conclusions

We have continued our studies on the purification and characterization of extracellular polysaccharides from *Burkholderia mallei*. In particular, we are tremendously excited about the use of extracellular polysaccharide components present on these organisms that may very well serve as ideal vaccine candidates. We have developed a model of experimental glanders in horses. Further, we have demonstrated that a capsule minus mutant of *B. mallei* is avirulent in the horse. The pathogenesis of disease due to *B. mallei* is complex and multifactorial; however, vaccine development for glanders is proceeding using capsule (PS), a major virulence determinant of *B. mallei*. Testing of rETA-PS conjugate vaccine against glanders in the horse model has the potential to allow us to draw parallels to the closely related disease, melioidosis, in humans. Numerous strategies for vaccine development exist including conjugate, DNA, attenuated and heterologous vaccines, all of which have demonstrated potential for further development. We must continue to study the underlying mechanisms of *B. mallei* pathogenesis to identify new potential targets for immunoprophylaxis. We have initiated studies using random promoter libraries and microarray analysis to examine the expression of virulence genes in *B. mallei*, and we have combined these studies with an examination of protein expression. The overall significance of the work resides in the realization that we are beginning to understand the virulence of *B. mallei*, and we are progressing towards the development of a vaccine that will protect against disease due to this organism.

Appendices

1. Burtnick, M.N., Brett, P.J., and Woods, D.E. 2002. Molecular and Physical Characterization of *Burkholderia mallei* O-Antigens. J. Bacteriol. 184:849-852.
2. Woods, D.E., Jeddeloh, J.A., Fritz, D.F., and DeShazer, D. 2002. *Burkholderia thailandensis* E125 Harbors a Temperate Bacteriophage Specific for *Burkholderia mallei*. J. Bacteriol. 184:4003-4017.
3. Brett, P.J., Burtnick, M.N., and Woods, D.E. 2003. The *wbiA* Locus is Required for 2-O-Acetylation of O-Antigens Expressed by *Burkholderia pseudomallei* and *Burkholderia thailandensis*. FEMS Microbiology Letters. In press.
4. Tribuddharat, C., Moore, R.A., Baker, P., and Woods, D.E. 2003. Characterization of Class A β-Lactamase Mutations of *Burkholderia pseudomallei* That Confer Selective Resistance Against Ceftazidime or Clavulanic Acid Inhibition. Antimicrobial Agents and Chemotherapy. In press.

APPENDIX 1

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Molecular and Physical Characterization of *Burkholderia mallei* O Antigens

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***Burkholderia mallei* lipopolysaccharide (LPS) has been previously shown to cross-react with polyclonal antibodies raised against *B. pseudomallei* LPS; however, we observed that *B. mallei* LPS does not react with a monoclonal antibody (Pp-PS-W) specific for *B. pseudomallei* O polysaccharide (O-PS). In this study, we identified the O-PS biosynthetic gene cluster from *B. mallei* ATCC 23344 and subsequently characterized the molecular structure of the O-PS produced by this organism.**

Burkholderia mallei is a gram-negative bacterium responsible for a disease known as glanders in solipeds and occasionally in humans (3, 8, 13). The factors involved in the pathogenesis of *B. mallei* infection remain relatively poorly defined at the molecular level. A previous study that identified a polysaccharide gene cluster in *B. mallei* showed that *B. mallei* lipopolysaccharide (LPS) cross-reacts with polyclonal antibodies raised against the LPS of *Burkholderia pseudomallei*, a closely related organism responsible for a disease known as melioidosis (6). In the present study, we investigated the LPS profiles of *B. mallei* strains, identified the gene cluster responsible for O polysaccharide (O-PS) biosynthesis in *B. mallei* ATCC 23344, and determined the physical structure of the *B. mallei* ATCC 23344 O-PS. Additionally, we showed that the O-PS moiety of *B. mallei* LPS is required for resistance to the bactericidal action of serum. Finally, we identified the presence of insertion sequences in two strains of *B. mallei* that disrupt the expression of O-PS.

Analysis of LPS profiles of *B. mallei* strains. The strains and plasmids used in this study are shown in Table 1. The first goal of this study was to assess the LPS profiles of *B. mallei* strains. Initially, we performed Western blot analysis of *B. mallei* ATCC 23344 whole-cell lysates with polyclonal rabbit sera raised against a *B. pseudomallei* bovine serum albumin (BSA)–O-PS conjugate as well as with a *B. pseudomallei* O-PS-specific MAb (Pp-PS-W) according to a previously described protocol (1, 2). As shown in Fig. 1A, *B. mallei* ATCC 23344 reacted with the anti-LPS polyclonal sera, resulting in a typical LPS banding pattern; however, the *B. pseudomallei* O-PS-specific MAb (Pp-PS-W) did not react. This indicated that differences exist between *B. mallei* and *B. pseudomallei* O-PS. We further assessed the LPS profiles of 10 different *B. mallei* strains (Fig. 1B). By using Western blot analysis, we showed that 8 of the 10 strains assessed bound the anti-LPS polyclonal sera and displayed typical LPS banding patterns. In contrast, however, two strains, NCTC 120 and ATCC 15310, did not bind the anti-LPS polyclonal sera, as indicated by the absence of bands (Fig. 1B). In order to confirm that the O-PS moiety was absent rather than a different type of O-PS, silver stain analysis was employed. Figure 1C shows the silver stain results confirming that both of these strains lacked O-PS moieties.

Identification and characterization of *B. mallei* ATCC 23344 O-PS biosynthetic gene cluster. In order to investigate the genes responsible for O-PS biosynthesis in *B. mallei*, we constructed a cosmid library by using *B. mallei* ATCC 23344 genomic DNA and the cosmid pScosBC1 by using a previously described protocol (12). Colony hybridizations were then performed with a 1.1-kb DNA fragment containing the recently identified *B. mallei wbiA* gene (P. Brett, M. Burtnick, and D. Woods, unpublished data). Six positive cosmid clones were obtained. Based on the *Bam*HI-*Kpn*I restriction patterns obtained, two cosmid clones, 1C3 and 2B5, were predicted to harbor the entire *B. mallei* O-PS gene cluster. Sequence analysis resulted in 19,918 bp of contiguous sequence containing the entire *B. mallei* O-PS biosynthetic gene cluster with an IS*407*-like insertion sequence element at the 3′ end.

The first 18,738 bp of the *B. mallei* DNA sequence contained 16 predicted ORFs that were identical to those previously defined as the O-PS biosynthetic gene cluster in *B. pseudomallei* (Fig. 2) (5). Sequence alignment of the *B. pseudomallei* and *B. mallei* O-PS biosynthetic regions revealed 99% identity at the nucleotide level. The genes comprising the *B. mallei* O-PS biosynthetic operon were named as per the identical genes found in *B. pseudomallei* (5).

Physical characterization of *B. mallei* O-PS moieties. In order to structurally analyze the *B. mallei* O-PS structure, it was necessary to construct a *B. mallei* strain unable to produce capsular polysaccharide (CPS), because CPS copurifies with LPS. The suicide vector pGSV3008 was employed as previously described to construct *B. mallei* PB100, a derivative of ATCC 23344 that does not produce CPS (6). The O-PS was purified as previously described for *B. pseudomallei*. Figure 3 shows ^{13}C nuclear magnetic resonance (^{13}C-NMR) analysis (Complex Carbohydrate Research Center, University of Georgia, Athens) results demonstrating that the *B. mallei* O-PS

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TABLE 1. Bacterial strains and cosmids or plasmids used in this study

Strain, cosmid, or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli		
SM10	Mobilizing strain: transfer genes of RP4 integrated into the chromosome; Km^r Sm^s	11
TOP10	High-efficiency transformation strain with blue/white screening; Ap^s Km^s	Invitrogen
HB101	Serum-sensitive strain	7
B. mallei		
NCTC 120		USAMRIID[a]
NCTC 10248	Human isolate	USAMRIID
NCTC 10229		USAMRIID
NCTC 10260	Human isolate	USAMRIID
NCTC 10247		USAMRIID
ATCC 23344	Human isolate; Pm^r Gm^s	USAMRIID
NCTC 3708	Mule isolate	USAMRIID
NCTC 3709	Horse isolate	USAMRIID
ATCC 10399		USAMRIID
ATCC 15310		USAMRIID
PB100	ATCC 23344::pGSV3008; Pm^r Gm^r	This study
B. pseudomallei 1026b	Clinical isolate; Gm^r Km^r Sm^r Pm^r Tp^s	4
Cosmids		
pScosBC1	Broad-host-range cosmid cloning vector based on pSuperCos1; Ap^r Tp^r	12
p1C3	pScosBC1 from ATCC 23344 library with a 23-kb fragment containing the O-PS biosynthetic gene cluster	This study
p2B5	pScosBC1 from ATCC 23344 library with a 27-kb fragment containing the O-PS biosynthetic gene cluster	This study
Plasmids		
pUC19	Cloning vector with blue/white selection; Ap^r	14
pGSV3008	pGSV containing a 379-bp *Eco*RI fragment from pDD3008, contains internal fragment from the *wcbB* gene; Gm^r	6

[a] U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Md.

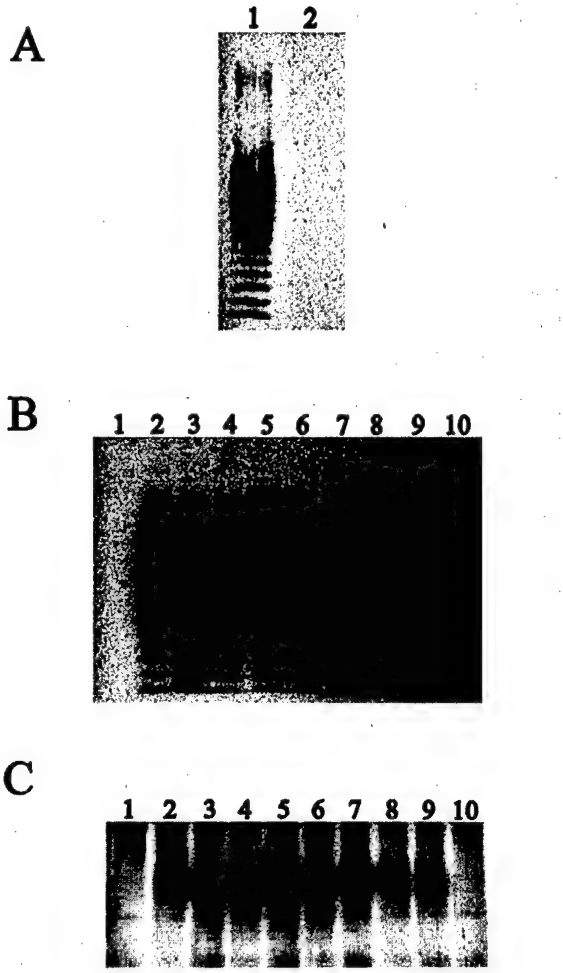

FIG. 1. (A) Western blot analysis of *B. mallei* ATCC 23344. Proteinase K-treated whole-cell lysates were used. In lane 1, the primary antibody used was a 1/2,000 dilution of polyclonal antisera raised against a *B. pseudomallei* BSA–O-PS conjugate, and in lane 2, the primary antibody used was a 1/2,000 dilution of the *B. pseudomallei* O-PS-specific MAb (Pp-PS-W). (B) Western blot profiles of proteinase K-treated whole-cell lysates of *B. mallei* strains. The primary antibody used was polyclonal sera raised against a *B. pseudomallei* BSA–O-PS conjugate. Lanes: 1, NCTC 120; 2, NCTC 10248; 3, NCTC 10229; 4, NCTC 10260; 5, NCTC 10247; 6, ATCC 23344; 7, NCTC 3708; 8, NCTC 3709; 9, ATCC 10399; and 10, ATCC 15310. (C) Silver stain analysis of proteinase K-treated whole-cell lysates of *B. mallei* strains. Lanes: 1, NCTC 120; 2, NCTC 10248; 3, NCTC 10229; 4, NCTC 10260; 5, NCTC 10247; 6, ATCC 23344; 7, NCTC 3708; 8, NCTC 3709; 9, ATCC 10399; and 10, ATCC 15310.

backbone is similar to that previously described for *B. pseudomallei* O-PS, a heteropolymer of repeating D-glucose and L-talose (9, 10). However, changes are apparent in the O-acetylation pattern of the *B. mallei* L-talose residue in comparison to that of *B. pseudomallei*. Similar to *B. pseudomallei* O-PS, *B. mallei* O-PS demonstrates the presence of *O*-acetyl or *O*-methyl substitutions at the 2′ position of the talose residue. In contrast, *B. mallei* O-PS is devoid of an *O*-acetyl group at the 4′ position of the talose residue. Thus, the structure of *B. mallei* O-PS is best described as 3)-β-D-glucopyranose-(1,3)-6-deoxy-α-L-talopyranose-(1-, in which the talose residue contains 2-*O*-methyl or 2-*O*-acetyl substituents. Recent studies indicate that the presence of 4-*O*-acetyl groups on the talose residues of *B. pseudomallei* O-PS is due to an O-acetylation locus unlinked to the previously described O-PS biosynthetic operon (Brett et al., unpublished). If this is the case, then the unlinked locus responsible for O-acetylation is either not present or is nonfunctional in *B. mallei* strains. The presence or absence of *O*-acetyl groups on the O-PS moieties may have consequences when O-PS is considered as a component of a vaccine that protects against both *B. mallei* and *B. pseudomallei*.

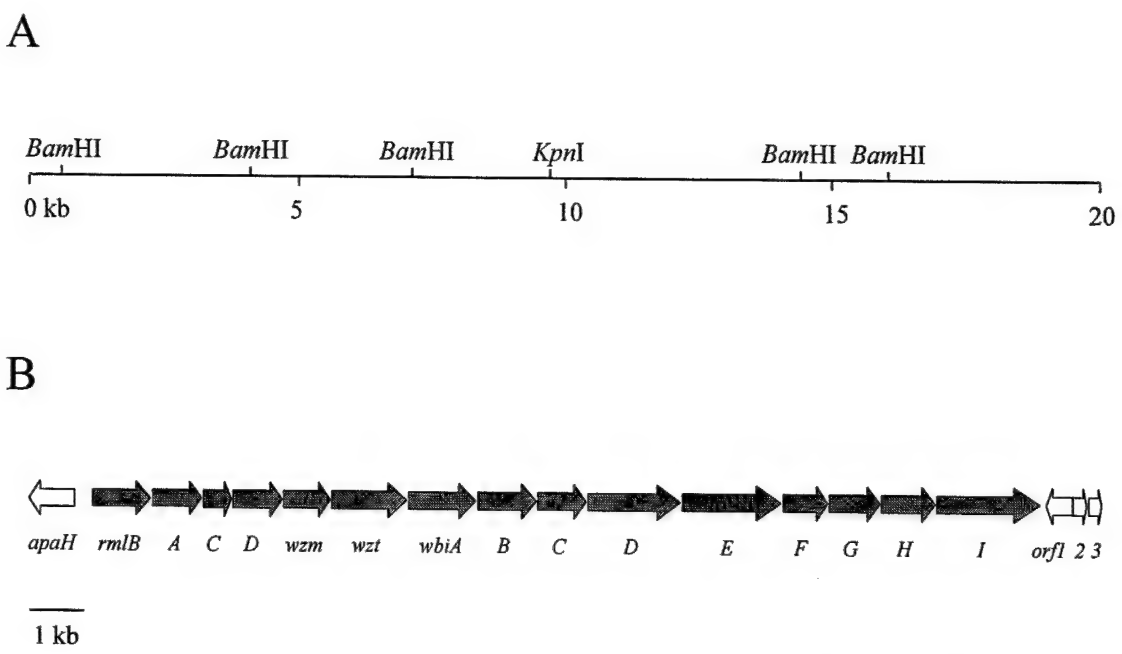

FIG. 2. Restriction and genetic maps of the *B. mallei* O-PS biosynthetic gene cluster. (A) Restriction map. The horizontal line represents the *B. mallei* DNA insert of cosmid 2B5. The locations of *Bam*HI and *Kpn*I cleavage sites used for subcloning are shown. Two additional *Bam*HI sites at the 5′ and 3′ ends of 2B5, which were part of the pScosBC1 vector, are not shown. (B) Genetic map. The location and direction of transcription of the genes are represented by arrows, and the gene names are shown below. The gray arrows indicate the genes involved in O-PS biosynthesis based on homology to the *B. pseudomallei* O-PS biosynthetic operon.

***B. mallei* survives in 30% NHS, and serum-sensitive strains lack the O-PS moiety of LPS.** The ability of *B. mallei* ATCC 23344 to grow in the presence of 30% normal human serum (NHS) was initially assessed with a serum bactericidal assay (5) in which viable counts were determined at 2, 4, 8, and 18 h. *B. mallei* ATCC 23344 was shown to survive in the presence of 30% NHS over the course of 18 h (Fig. 4A). Serum-resistant *B. pseudomallei* 1026b and serum-sensitive *Escherichia coli* HB101 were employed as controls.

In order to assess the role of *B. mallei* O-PS in serum resistance, NHS bactericidal assays (5) were performed with *B. mallei* ATCC 23344 and *B. mallei* NCTC 120 and ATCC 15310, the two strains lacking O-PS. *B. mallei* ATCC 23344 remained resistant to the killing action of 30% NHS, while NCTC 120 and ATCC 15310 were completely killed following a 2-h incubation in 30% NHS (Fig. 4B). The other seven *B. mallei* strains used in this study possessed intact O-PS moieties and were resistant to the bactericidal action of 30% NHS (data not shown). These results suggested that *B. mallei* O-PS moieties play a crucial role in the serum resistance of this organism: this correlates well with previous studies demonstrating that *B. pseudomallei* O-PS is required for serum resistance (5).

Identification of insertion sequence IS*407* in the O-PS biosynthetic gene clusters of *B. mallei* NCTC 120 and ATCC 15310. In order to determine if the O-PS biosynthetic gene clusters of NCTC 120 and ATCC 15310 had been disrupted, we chose to individually PCR amplify each gene present in this cluster. Deoxyoligonucleotide primers were designed outside of the 5′ and 3′ ends of each gene. *B. mallei* ATCC 23344 chromosomal DNA was used a control as an indicator of the size of a wild-type copy of each gene. Alterations were observed in the *wbiE* PCR product from NCTC 120 and in the *wbiG* PCR product from ATCC 15310. The PCR products obtained in both cases were approximately 1.5 kb larger than those obtained with ATCC 23344 genomic DNA (data not shown). Cloning and sequence analysis of the NCTC 120 *wbiE* and ATCC 15310 *wbiG* PCR products revealed the presence of insertion sequences within these two genes. In NCTC 120, an IS*407*-like element was located after nucleotide 13615 of the O-PS operon in the *wbiE* gene. In ATCC 15310, an IS*407*-like element was located following nucleotide 15107 of the O-PS operon in the *wbiG* gene. It is likely that the presence of insertion elements in the O-PS biosynthetic gene clusters of *B. mallei* NCTC 120 and ATCC 15310 is responsible for the loss of expression of O-PS in these two strains. DeShazer et al. have previously demonstrated the presence of an IS*407*-like element (termed "IS*407A*") at the 3′ end of the CPS gene cluster and have shown that this element is active in *B. mallei* (6). The data presented in this paper certainly support the view that IS*407* is functionally active in *B. mallei*.

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We are grateful to Patricia Baker and Francois Becotte for excellent technical assistance. We thank David DeShazer for providing us with the plasmid pGSV3008.

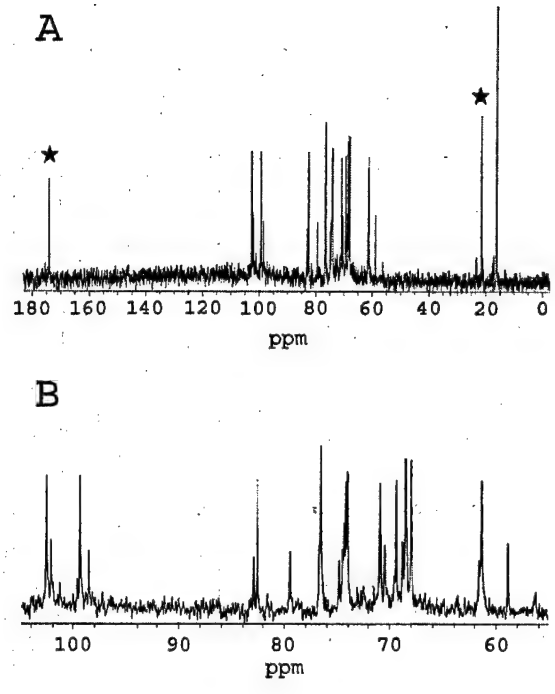

FIG. 3. ^{13}C-NMR analysis of *B. mallei* PB100 O-PS. (A) *O*-Acetyl peaks are indicated by stars. (B) Expanded view of the region running from 100 to 60 ppm. (C) Structure of *B. pseudomallei* and *B. mallei* O-PS. In *B. pseudomallei*, in 33% of the talose residues, R′ = *O*-methyl and R″ = *O*-acetyl, and in 66% of the talose residues, R′ = *O*-acetyl and R″ = OH. In *B. mallei*, R′ = *O*-acetyl or *O*-methyl and R″ = OH.

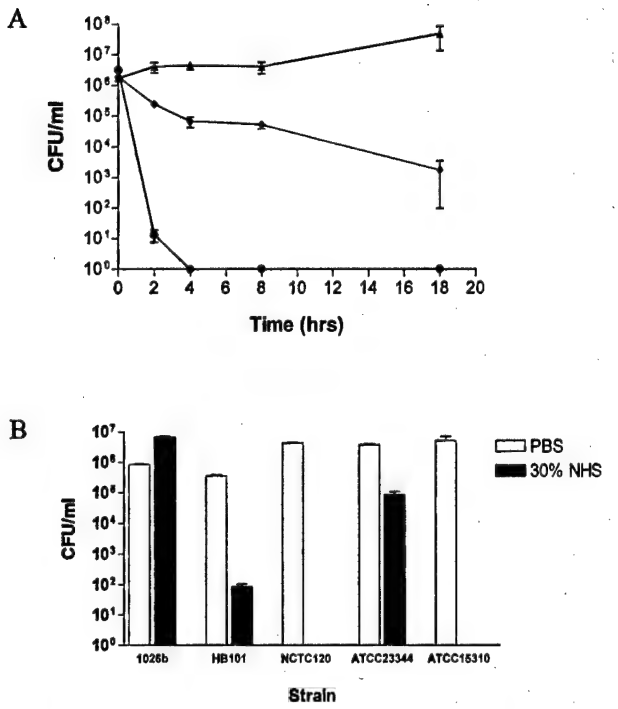

FIG. 4. Serum bactericidal assays with *B. mallei* strains. (A) Thirty percent NHS killing assay in which viable counts were determined at the 2-, 4-, 8-, and 18-h time points. *B. pseudomallei* 1026b (▲), *B. mallei* ATCC 23344 (◆), and *E. coli* HB101 (●). (B) Thirty percent NHS killing assay in which viable counts were determined following a 2-h incubation at 37°C. Control tubes containing phosphate-buffered saline (PBS) are shown as white bars, and experimental tubes containing 30% NHS are shown as gray bars. Error bars indicate standard deviations.

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APPENDIX 2

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Burkholderia thailandensis E125 Harbors a Temperate Bacteriophage Specific for *Burkholderia mallei*

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***Burkholderia thailandensis* is a nonpathogenic gram-negative bacillus that is closely related to *Burkholderia mallei* and *Burkholderia pseudomallei*. We found that *B. thailandensis* E125 spontaneously produced a bacteriophage, termed φE125, which formed turbid plaques in top agar containing *B. mallei* ATCC 23344. We examined the host range of φE125 and found that it formed plaques on *B. mallei* but not on any other bacterial species tested, including *B. thailandensis* and *B. pseudomallei*. Examination of the bacteriophage by transmission electron microscopy revealed an isometric head and a long noncontractile tail. *B. mallei* NCTC 120 and *B. mallei* DB110795 were resistant to infection with φE125 and did not produce lipopolysaccharide (LPS) O antigen due to IS*407*A insertions in *wbiE* and *wbiG*, respectively. *wbiE* was provided in *trans* on a broad-host-range plasmid to *B. mallei* NCTC 120, and it restored LPS O-antigen production and susceptibility to φE125. The 53,373-bp φE125 genome contained 70 genes, an IS*3* family insertion sequence (IS*Bt3*), and an attachment site (*attP*) encompassing the 3′ end of a proline tRNA (UGG) gene. While the overall genetic organization of the φE125 genome was similar to λ-like bacteriophages and prophages, it also possessed a novel cluster of putative replication and lysogeny genes. The φE125 genome encoded an adenine and a cytosine methyltransferase, and purified bacteriophage DNA contained both N6-methyladenine and N4-methylcytosine. The results presented here demonstrate that φE125 is a new member of the λ supergroup of *Siphoviridae* that may be useful as a diagnostic tool for *B. mallei*.**

The disease glanders is caused by *Burkholderia mallei*, a host-adapted pathogen that does not persist in nature outside of its horse host (32). Glanders is a zoonosis, and humans whose occupations put them into close contact with infected animals can contract the disease. There have been no naturally occurring cases of glanders in North America in the last 60 years, but laboratory workers are still at risk of infection with *B. mallei* via cutaneous (68) and inhalational (31) routes. Human glanders has been described as a painful and loathsome disease from which few recover without antibiotic intervention (33, 51). There is little known about the virulence factors of this organism, but a recent report indicates that the capsular polysaccharide is essential for virulence in hamsters and mice (24).

Burkholderia pseudomallei is the etiologic agent of the glanders-like disease melioidosis (21). As the names suggest, *B. mallei* and *B. pseudomallei* are closely related species (19, 56, 59, 69). These β-*Proteobacteria* can now be directly compared at the genomic level because the *B. pseudomallei* K96243 genomic sequence is available at the Sanger Institute website (http://www.sanger.ac.uk/) and the *B. mallei* ATCC 23344 genomic sequence is available at the TIGR (The Institute for Genomic Research) website (http://www.tigr.org/). Preliminary BLAST (4) comparisons indicate that the genes conserved between these species are ~99% identical at the nucleotide level. This high level of nucleotide identity makes it challenging to use nucleic acid-based assays to discriminate between *B. mallei* and *B. pseudomallei* (6, 71).

There are legitimate concerns that *B. mallei* and *B. pseudomallei* may be misused as biological weapons (16, 46, 51, 60), and there is compelling evidence that *B. mallei* has already been used in this manner (3, 74). Diagnostic assays should be developed to discriminate between these microorganisms in the event that they are misused in the future. The use of a combination of diagnostic assays may be necessary to discriminate between these species, including nucleic acid-based assays, phenotypic assays (colony morphology, motility, and carbohydrate utilization), enzyme-linked immunosorbent assay, intact cell matrix-assisted laser desorption ionization–time of flight, and bacteriophage susceptibility.

In 1957 Smith and Cherry described eight lysogenic *B. pseudomallei* strains that produced bacteriophage that were more active on *B. mallei* than on *B. pseudomallei* (67). In fact, bacteriophage E attacked *B. mallei* strains exclusively. Manzenink et al. (45a) found that 91% of their *B. pseudomallei* strains were lysogenic and that three bacteriophages, PP19, PP23, and PP33, could be used in combination to identify *B. mallei*. Unfortunately, these *B. mallei*-specific bacteriophage were not further characterized and are not readily available. It is interesting that neither study identified bacteriophage production by *B. mallei* strains.

The purpose of this work was to identify and characterize a *B. mallei*-specific bacteriophage and make it available to the scientific community. *Burkholderia thailandensis* is a nonpathogenic soil saprophyte that has been described as *B. pseudomallei*-like (9, 10), and there are no published reports describing bacteriophage production by this species. *B. thailandensis*

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TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics[a]	Source or reference
pBluescript KS	General cloning vector; ColE1; Ap^r	Stratagene
pDW1	pBluescript KS containing 1,068-bp *Hin*dIII fragment from ϕE125	This study
pDW3.2	pBluescript KS containing 3,231-bp *Hin*dIII fragment from ϕE125	This study
pDW4.4	pBluescript KS containing 4,351-bp *Hin*dIII fragment from ϕE125	This study
pDW5.5	pBluescript KS containing 5,448-bp *Hin*dIII fragment from ϕE125	This study
pDW7.5	pBluescript KS containing 7,325-bp *Hin*dIII fragment from ϕE125	This study
pDW9.5	pBluescript KS containing 9,025-bp *Hin*dIII fragment from ϕE125	This study
pDW11	pBluescript KS containing 9,942-bp *Hin*dIII fragment from ϕE125	This study
pDW18	pBluescript KS containing 12,983-bp *Hin*dIII fragment from ϕE125	This study
pSKM11	Positive selection cloning and suicide vector; IncP oriT; ColE1 ori; Ap^r Tc^s	50
pSKM3.2	pSKM11 containing 3,231-bp *Hin*dIII fragment from ϕE125; Ap^r Tc^r	This study
pDD5003B	37.4-kb *Bam*HI fragment from DD5003 obtained by self-cloning; Ap^r Tc^r	This study
pCR2.1	3.9-kb TA cloning vector; pMB1 oriR; Km^r Ap^r	Invitrogen
pAM1	pCR2.1 containing ϕE125 gene*27* downstream of the *lac* promoter	This study
pCM1	pCR2.1 containing ϕE125 gene*56* downstream of the *lac* promoter	This study
pDD70	pCR2.1 containing 3.2-kb NCTC 120 *wbiE*::IS*407*A PCR fragment	This study
pDD71	pCR2.1 containing 3.2-kb DB110795 *wbiG*::IS*407*A PCR fragment	This study
pDD72	pCR2.1 containing ATCC 23344 *wbiE*	This study
pSPORT 1	General cloning vector; ColE1; Ap^r	Life Technologies
pSPORT 8.1	pSPORT 1 containing 8.1-kb *Hin*dIII-*Eco*RI fragment from pDD5003B	This study
pBHR1	Mobilizable broad-host-range vector; Km^r Cm^r	MoBiTec
pBHR1-*wbiE*	pBHR1 containing 1.8-kb *Eco*RI fragment from pDD72; Km^r Cm^s	This study

[a] Abbreviations: Ap, ampicillin; Tc, tetracycline; Km, kanamycin; Cm, chloramphenicol.

E125, isolated in 1991 from soil in northeastern Thailand (70), spontaneously produced a temperate bacteriophage (ϕE125) that attacked *B. mallei* but not any other bacterial species examined. The gene order and modular organization of the ϕE125 genome is reminiscent of lambdoid bacteriophages (11, 34), and it contains several interesting features, including an insertion sequence, two DNA methylase genes, and a novel cluster of putative replication and lysogeny genes. Bacteriophage ϕE125 exhibits a B1 morphotype and therefore is a new member of the family *Siphoviridae* (phage with long noncontractile tails) (1, 2).

MATERIALS AND METHODS

Bacterial plasmids, strains, and growth conditions. The plasmids used in this study are described in Table 1. The *B. mallei* strains used in this study are listed in Table 2. The following *B. pseudomallei* strains were used in this study: 316c, NCTC 4845, 1026b, WRAIR 1188, USAMRU Malaysia 32, Pasteur 52237, STW 199-2, STW 176, STW 115-2, STW 152, STW 102-3, STW 35-1, K96243, 576a, 275, 295, 296, 503, 506, 112c, 238, 423, 465a, 776, 439a, 487, 644, 713, 730, E8, E12, E13, E24, E25, E40, E203, E210, E214, E215, E250, E272, E277, E279, E280, E283, E284, E300, E301, E302, and E304 (5, 20, 22, 25, 26, 66, 76). *B. thailandensis* strains E27, E30, E32, E96, E100, E105, E111, E120, E125, E132, E135, E202, E251, E253, E254, E255, E256, E257, E258, E260, E261, E263, E264, E266, E267, E275, E285, E286, E290, E293, E295, and E299 (10, 66, 76) were also utilized in this study. Other *Burkholderia* species used in this study include *B. cepacia* LMG 1222 (genomovar I) (44), *B. multivorans* C5568, *B. multivorans* LMG 18823 (44), *B. cepacia* LMG 18863 (genomovar III) (44), *B. cepacia* 715j (genomovar III) (47), *B. stabilis* LMG 07000, *B. vietnamiensis* LMG 16232 (44), *B. vietnamiensis* LMG 10929 (44), *B. gladioli* 2-72 (62), *B. gladioli* 2-75 (62), *B. gladioli* 4-54 (62), *B. gladioli* 5-62 (62), *B. uboniae* EY 3383 (77), *B. cocovenans* ATCC 33664, *B. pyrrocinia* ATCC 15958, *B. glathei* ATCC 29195, *B. caryophylli* Pc 102, *B. andropogonis* PA-133, *B. kururiensis* KP23 (79), *B. sacchari* IPT101 (8), *Burkholderia* sp. strain 2.2N (13), and *Burkholderia* sp. strain T-22-8A. *Ralstonia solanacearum* FC228, *R. solanacearum* FC229, *R. solanacearum* FC230, *Pandoraea apista* LMG 16407 (17), *Pandoraea norimbergensis* LMG 18379 (17), *Pandoraea pnomenusa* LMG 18087 (17), *Pandoraea pulmonicola* LMG 18106 (17), *Stenotrophomonas maltophilia* XM16 (39), *S. maltophilia* XM47 (39), *Pseudomonas aeruginosa* PAO (30), *P. aeruginosa* PA14 (55), *Pseudomonas syringae* DC3000 (73), *Salmonella enterica* serovar Typhimurium SL1344 (29), *Serratia marcescens* H11, *Escherichia coli* TOP10 (Invitrogen), S17-1λ*pir* (65), HB101 (7), MC4100 (15), DH5α (Gibco BRL), JM105 (78), E2348/69 (41), and DB24 (36) were also used in this study. *E. coli* was grown at 37°C on Luria-Bertani (LB) agar (Lennox) or in LB broth (Lennox). *P. syringae*, *B. andropogonis*, *Burkholderia* sp. strain 2.2N, *Burkholderia* sp. strain T-22-8A, *B. glathei*, and *B. caryophylli* were grown at 25°C on LB agar or in LB broth containing 4% glycerol. All other bacterial strains were grown at 37°C on LB agar or in LB broth containing 4% glycerol. When appropriate, antibiotics were added at the following concentrations: 100 μg of ampicillin, 30 μg of chloramphenicol, 25 μg of kanamycin, and 15 μg of tetracycline per ml for *E. coli* and 100 μg of streptomycin and 50 μg of tetracycline per ml for *B. thailandensis*. *B. mallei* DD3008 was grown in the presence of 5 μg of gentamicin per ml, and *B. mallei* NCTC 120 (pBHR1) was grown in the presence of 15 μg of polymyxin B and 5 μg of kanamycin per ml.

Spontaneous bacteriophage production by lysogenic *B. thailandensis* strains and UV induction experiments. *B. thailandensis* strains E264, E275, E202, E125, and E251 were grown in LB broth for 18 h at 37°C with shaking (250 rpm). One hundred microliters of each saturated culture was used to inoculate two LB broth (3-ml) subcultures. One set of subcultures was incubated for 5 h under the same conditions. The other set of subcultures was incubated for 3 h, poured into sterile petri dishes in a class II biological safety cabinet, subjected to a hand-held UV light source (254 nm) for 20 s (25 cm above the sample), pipetted back into culture tubes, and incubated for an additional 2 h. Both sets of subcultures were briefly centrifuged to pellet the cells, and the supernatants were filter sterilized (0.45-μm-pore-size filters). The samples were serially diluted in suspension medium (SM) (40), and the numbers of PFU were assessed by using *B. mallei* ATCC 23344 as the host strain as described below. Bacteriophage was considered to be induced if the titer increased twofold (or more) after exposure to UV light. If bacteriophage titers did not increase twofold, the bacteriophage was not considered to be induced by UV light.

Bacteriophage ϕE125 propagation and DNA purification. The protocols followed for picking plaques, titrating bacteriophage stocks, and preparing plate lysates were the same as those used for bacteriophage λ (61), with a few minor modifications. Briefly, 0.1 ml of ϕE125 and 0.1 ml of a saturated culture of *B. mallei* ATCC 23344 (~5×10^8 bacteria) were mixed and incubated at 25°C for 20 min, and 4.8 ml of molten LB top agar (0.7%) containing 4% glycerol was added. The mixture was immediately poured onto LB plates containing 4% glycerol and incubated overnight at 37°C. For preparation of plate lysate stocks, 5 ml of SM was added to the plate, and bacteriophage was eluted overnight at 4°C without shaking. SM was harvested, bacterial debris was separated by centrifugation, and the resulting supernatant was filter sterilized (0.45-μm-pore-size filters) and stored at 4°C. Bacteriophage ϕE125 DNA was purified from a plate culture lysate using the Wizard Lambda Preps DNA Purification System (Promega). The ϕE125 lysogen BML10 was isolated from a single turbid plaque

TABLE 2. Bacteria used to examine the host range of bacteriophage φE125

Bacterium	Relevant information	Plaque formation[a]
Burkholderia mallei		
NCTC 120	LPS O-antigen mutant; *wbiE*::IS*407*A	−
NCTC 10248		+
NCTC 10229		+
NCTC 10260		+
NCTC 10247		+
NCTC 3708		+
NCTC 3709		+
ATCC 23344		+
ATCC 10399		+
ATCC 15310		+
DB110795	Laboratory-passaged ATCC 15310; LPS O-antigen mutant; *wbiG*::IS*407*A	−
BML10	ATCC 23344 (φE125)	−
DD3008	ATCC 23344::pGSV3008; capsule mutant	+
Burkholderia pseudomallei	50 strains	−
Burkholderia thailandensis	32 strains	−
Burkholderia cepacia	Genomovar I; 1 strain	−
Burkholderia multivorans	2 strains	−
Burkholderia cepacia	Genomovar III; 2 strains	−
Burkholderia stabilis	1 strain	−
Burkholderia vietnamiensis	2 strains	−
Burkholderia gladioli	4 strains	−
Burkholderia uboniae	1 strain	−
Burkholderia cocovenans	1 strain	−
Burkholderia pyrrocinia	1 strain	−
Burkholderia glathei	1 strain	−
Burkholderia caryophylli	1 strain	−
Burkholderia andropogonis	1 strain	−
Burkholderia kururiensis	1 strain	−
Burkholderia sacchari	1 strain	−
Burkholderia spp.	2 strains	−
Ralstonia solanacearum	3 strains	−
Pandoraea apista	1 strain	−
Pandoraea norimbergensis	1 strain	−
Pandoraea pnomenusa	1 strain	−
Pandoraea pulmonicola	1 strain	−
Stenotrophomonas maltophilia	2 strains	−
Pseudomonas aeruginosa	2 strains	−
Pseudomonas syringae	1 strain	−
Salmonella enterica serovar Typhimurium	1 strain	−
Serratia marcescens	1 strain	−
Escherichia coli	8 strains	−

[a] +, present; −, absent.

formed on ATCC 23344. The plaque was picked with a Pasteur pipette, transferred to a tube containing 3 ml of broth media, and incubated overnight. The saturated culture was spread onto solid media with an inoculating loop, and 10 isolated colonies were tested for their ability to form plaques with φE125. All of the colonies were resistant to infection with φE125, and one was selected and designated BML10.

φE125 sensitivity testing. Approximately 10^2 PFU was added to a saturated bacterial culture and incubated at 25°C for 20 min, and 4.8 ml of molten LB top agar (0.7%) containing 4% glycerol was added. The mixture was immediately poured onto a LB plate containing 4% glycerol and incubated overnight at 25 or 37°C, depending on the bacterial species being tested. Bacteria were considered to be sensitive to φE125 if they formed plaques under these conditions and resistant if they did not. It should be noted that the positive control, *B. mallei* ATCC 23344, formed plaques in the presence of φE125 after incubation at 25 and 37°C. No bacterial species tested formed plaques in the absence of φE125.

Negative staining of φE125. Bacteriophage φE125 was prepared from 20 ml of a plate culture lysate (see above), incubated at 37°C for 15 min with Nuclease Mixture (Promega), precipitated with Phage Precipitant (Promega), and resuspended in 1 ml of Phage Buffer (Promega). The bacteriophage solution (~100 μl) was added to a strip of parafilm M (Sigma), and a formvar-coated nickel grid (400 mesh) was floated on the bacteriophage solution for 30 min at 25°C. Excess fluid was removed, and the grid was placed on a drop of 1% phosphotungstic acid, pH 6.6, for 2 min at 25°C. Excess fluid was removed, and the specimen was examined on a Philips CM100 transmission electron microscope. Nickel grids were glow discharged on the day of use.

DNA manipulation and plasmid conjugation. Restriction enzymes and T4 DNA ligase were purchased from Roche Molecular Biochemicals and were used according to the manufacturer's instructions. DNA fragments used in cloning procedures were excised from agarose gels and purified with a GeneClean III Kit (Bio 101). Bacterial genomic DNA was prepared by using the Masterpure DNA Kit (Epicentre) for methylase dot blot assays and by a previously described protocol (75) for all other experiments. Plasmids were purified from overnight cultures using Wizard *Plus* SV Minipreps (Promega). The broad-host-range plasmids pBHR1 and pBHR1-*wbiE* were electroporated into *E. coli* S17-1λ*pir* (12.25 kV/cm) and conjugated to *B. mallei* NCTC 120 for 8 h as described elsewhere (22). Similarly, the suicide vector pSKM3.2 was electroporated into *E. coli* S17-1λ*pir* and conjugated to *B. thailandensis* E125 for 8 h as described elsewhere (22). The resulting strain, *B. thailandensis* DD5003, contained pSKM3.2 integrated into the φE125 genome at the 3.2-kb *Hin*dIII fragment. Chromosomal DNA was isolated from DD5003 and digested with the restriction endonuclease *Bam*HI, and the bacteriophage attachment site and flanking bacterial DNA were obtained by self-cloning (22).

Immunoblot analysis. Fifty microliters of a saturated broth culture of *B. mallei* was subjected to centrifugation, and the bacterial pellet was washed with phosphate-buffered saline, pH 7.4. The sample was resuspended in 50 μl of sample buffer (4% sodium dodecyl sulfate [SDS], 10% glycerol, 5% 2-mercaptoethanol,

0.005% bromphenol blue in Tris buffer, pH 6.8) and boiled for 10 min. The sample was treated with proteinase K (25 μg dissolved in 10 μl of sample buffer) and incubated at 37°C for 1 h. Forty microliters of sample was boiled for 5 min, loaded onto a 4% polyacrylamide stacking gel–12% polyacrylamide separating gel, and SDS-polyacrylamide gel electrophoresis (PAGE) was performed using 1× Tris-Glycine SDS Running buffer (Novex). The gel was blotted to Immuno-Blot PVDF Membrane (Bio-Rad) by using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) according to the manufacturer's instructions. The membrane was subjected to a blocking step (5% skim milk, 0.1% Tween 20) and was reacted with a 1:2,000 dilution of 3D11, a monoclonal mouse immunoglobulin G1 (IgG1) antibody that reacts with *B. mallei* lipopolysaccharide (LPS) O antigen (Research Diagnostics, Inc.). Following several washing steps with blocking buffer, the membrane was reacted with a 1:5,000 dilution of peroxidase-labeled goat antibody to mouse IgG (Kirkegaard & Perry Laboratories, Inc. [KPL]). Finally, it was washed three times with blocking buffer and once with PBS (pH 7.4) and then incubated with TMB Membrane Peroxidase Substrate (KPL).

DNA sequencing and analysis. DNA sequencing was performed at ACGT, Inc. (Northbrook, Ill.) and at LMT Sequencing Lab (Frederick, Md.). Most ϕE125 genes were identified by using GeneMark.hmm (43), whereas others were identified by visual inspection, guided by BLAST (4) results. DNA and protein sequences were analyzed with GeneJockeyII and MacVector 7.1 software for the Macintosh. The gapped BLASTX and BLASTP programs were used to search the nonredundant sequence database for homologous proteins (4). In order to determine the nucleotide sequence of the ϕE125 *cos* sites, we sequenced the ends of ϕE125 DNA directly by using the following primers: COS4, 5′-AATCC GGCTCGTCCTTATTC-3′ and COS10, 5′-GTTGCGGTGACGTGGTGGTG-3′. The nucleotide sequences obtained contained a gap relative to the ligated ϕE125 ends on pDW9.5, which corresponded to unsequenceable 3′ ends (64).

PCR amplifications. PCR products were sized by using agarose electrophoresis and cloned using the pCR2.1 TOPO TA cloning kit (Invitrogen) and chemically competent *E. coli* TOP10 (Invitrogen). PCR amplifications were performed with a final reaction volume of 100 μl and contained 1× *Taq* PCR Master Mix (Qiagen), 1 μM oligodeoxyribonucleotide primers, and approximately 200 ng of genomic DNA. PCR mixtures were transferred to a PTC-150 MiniCycler with a Hot Bonnet accessory (MJ Research) and heated to 97°C for 5 min. This was followed by 30 cycles of a three-temperature cycling protocol (97°C for 30 s, 55°C for 30 s, and 72°C for 2 min) and one cycle at 72°C for 10 min. The eight oligodeoxyribonucleotide primer pairs used in the PCR amplification of the LPS O-antigen gene cluster were as follows: 1-1, 5′-CGAGTTCACGGTATCACAA G-3′, and 1-2, 5′-GTTGTCGTAGAAGTACAGCC-3′; 2-1, 5′-GGCTGTACTT CTACGACAAC-3′, and 2-2, 5′-GCATCAGCAGCGGATTGAAG-3′; 3-1, 5′-CTTCAATCCGCTGCTGATGC-3′, and 3-2, 5′-GAATGCGACTTCAACAAC AC-3′; 4-1, 5′-GTGTTGTTGAAGTCGCATTC-3′, and 4-2, 5′-CATAAACGT TCTGCAGACGC-3′; 5-1, 5′-GCGTCTGCAGAACGTTTATG-3′, and 5-2, 5′-GATTTGCTGCAAATAGCGTG-3′; 6-1, 5′-CACGCTATTTGCAGCAAAT C-3′, and 6-2, 5′-CGAAGATATCGAGCCAGTGC-3′; 7-1, 5′-GCACTGGCTC GATATCTTCG-3′, and 7-2, 5′-CCGAAGCGGTTGAAGAAGTG-3′; 8-1A, 5′-CTGGAAATGGCTATGAGCAG-3′, and 8-2A, 5′-AAATGCTCGCGTCATG TTGC-3′.

In order to determine the order and orientation of the *Hin*dIII fragments in the intact ϕE125 genome, outward-oriented primers specific for the ends of each *Hin*dIII fragment (except the 1,068-bp fragment) were synthesized and PCR was performed with ϕE125 genomic DNA and all possible primer combinations. We reasoned that two *Hin*dIII fragments were adjacent if we obtained a PCR product with primer pairs specific for the corresponding ends of those fragments. All PCR products were cloned and sequenced to confirm the PCR results. For these PCRs, and all of the PCR experiments mentioned below, the conditions mentioned above were used, with the following exception: we used 72°C for 30 s instead of 72°C for 2 min in the three-temperature cycling protocol. The 14 oligodeoxyribonucleotide primers used in this analysis were as follows: 3.2F, 5′-AGACGATCAAGCAACACGAG-3′; 3.2R, 5′-TCGAAGCGCCAATAAA ACGC-3′; 4.4F, 5′-CAAGCTCTCTCAGCTTCTCG-3′; 4.4R, 5′-ACCAGCGG CCATACATTATG-3′; 5.5F, 5′-GGTCTCCGGATCGTAATTGT-3′; 5.5R, 5′-TCGTGCGTCAGTTCAAATGG-3′; 7.5F, 5′-CCAGATCCAGAATACGCAA C-3′; 7.5R, 5′-ATAACGCGCTTTGTCGATCG-3′; 9.5F, 5′-GAGTGAAGCCA TCGAAGATC-3′; 9.5R, 5′-ACGGAAAGGAGCATGTCATC-3′; 11F, 5′-TCA TCGACGAGGAACTTCAC-3′; 11R, 5′-AATGATGGTCAGCACGAACG-3′; 18F-2, 5′-TCAAGGTAGAACAGCGTGTG-3′; 18R, 5′-GCTCCTTGTCCAA GTAGATG-3′.

PCR was performed with genomic DNAs from *B. mallei* ATCC 23344, *B. mallei* BML10, ϕE125, and the primers Pro (5′-TATACCCGACCGAATTGG-3′) and Int (5′-TATGACGTGAAGGCACTC-3′) to determine if ϕE125 integrated into the proline tRNA (UGG) gene in *B. mallei*. We obtained a single PCR product of the expected size (550 bp) with *B. mallei* BML10 DNA. This product was cloned, and its nucleotide sequence was determined. No PCR products were obtained when genomic DNAs from *B. mallei* ATCC 23344 or ϕE125 were used in the PCR.

Genomic DNA from ϕE125 was used for PCR amplification of gene*27* with the following primers: AM-UP, 5′-CAAGTTTAAAAACGGCTTTCAC-3′, and AM-DOWN, 5′-CAGCCAATCGATCAGAACAG-3′. The resulting PCR product was cloned, sequenced, and designated pAM1 (Table 1). Similarly, gene*56* was amplified by PCR using ϕE125 genomic DNA and the following primers: CM-UP, 5′-CACAGGTGCTGTTCAATCTC-3′, and CM-DOWN, 5′-CTCAC ATGACCTCCAAAACG-3′. The resulting PCR product was cloned, sequenced, and designated pCM1 (Table 1).

Dot blot assay for DNA methyltransferase activity. The plasmids pCR2.1, pAM1, and pCM1 (Table 1) were electroporated into *E. coli* DB24, a strain that lacks all endogenous DNA methylation (36). The transformants were grown overnight in the presence of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and genomic DNA was isolated as described above. Genomic DNA preparations were diluted in Tris-EDTA buffer (10 mM Tris-Cl [pH 7.4], 1 mM EDTA [pH 8.0]) to yield stocks of 150, 50, and 15 ng/μl, and 3-μl aliquots of each were spotted onto a BA85 nitrocellulose filter (Schleicher & Schuell). Methylase activity was assessed by using rabbit primary antibodies that react specifically with DNA containing N6-methyladenine (m6A) or N4-methylcytosine (m4C) in a dot blot assay, as described previously (36). The secondary antibody was a peroxidase-labeled goat anti-rabbit IgG (H + L) conjugate (KPL). Primary and secondary antibodies were used at dilutions of 1:50,000 and 1:1,000, respectively. Detection was accomplished by using the luminol system (Amersham/Pharmacia), and exposures were made to hyperfilm-ECL (Amersham/Pharmacia). The film images were digitally captured using a UMAX flatbed scanner (S900) and Adobe Photodeluxe (version 1.1) software for the PowerMac.

GenBank and American Type Culture Collection (ATCC) accession numbers. The nucleotide sequences reported in this paper were deposited in the GenBank database under the accession numbers AF447491 (ϕE125 genome) and AY063741 (*B. thailandensis* bacteriophage attachment site). ϕE125 was deposited in the ATCC bacteriophage collection and was assigned the accession number ATCC 23344-B1.

RESULTS

***B. thailandensis* strains spontaneously produce bacteriophage that infect *B. mallei*.** Five strains of *B. thailandensis* (E125, E202, E251, E264, and E275) were examined for the production of *B. mallei*-specific bacteriophage. All of the strains, with the exception of E251, spontaneously produced bacteriophage that formed plaques with diameters of 1.5 to 2 mm on *B. mallei* ATCC 23344. Strain E264 produced two bacteriophages that formed distinct plaques, one turbid and one clear. Strains E125, E202, and E275 each produced a bacteriophage that formed turbid plaques. Bacteriophage production was increased 2-fold (E264 and E275), 6-fold (E125), and 55-fold (E202) by brief exposure to UV light. The clear plaque bacteriophage from E264 was not induced, and UV light did not induce bacteriophage production by E251. We examined the host range of all five *B. thailandensis* bacteriophages on 10 strains of *B. mallei* and 13 strains of *B. pseudomallei* and found that the temperate bacteriophages produced by E264, E202, and E275 formed plaques on 9 of 10 *B. mallei* strains and on 3 of 13 *B. pseudomallei* strains. Since these bacteriophages were not specific for *B. mallei*, they were not further characterized. The clear plaque bacteriophage produced by E264 (LPE264) and the temperate bacteriophage produced by E125 (ϕE125) formed plaques on 8 of 10 and 9 of 10 *B. mallei* strains, respectively. Neither bacteriophage formed plaques on *B. pseudomallei* or on *B. mallei* NCTC 120. Typical yields of plate lysate stocks of LPE264 were 10^5 PFU/ml, and yields of ϕE125 were 10^8 PFU/ml. Bacteriophage

LPE264 was not further characterized in this study due to its low yield and its inability to form plaques on *B. mallei* NCTC 3709. Taken together, these results indicate that lysogenic *B. thailandensis* strains exist in nature and that the bacteriophage they harbor are spontaneously produced and infect *B. mallei*.

Bacteriophage ϕE125 is *B. mallei* specific. The host range of ϕE125 was examined with 139 bacterial strains, including 13 strains of *B. mallei*, 50 strains of *B. pseudomallei*, and 32 strains of *B. thailandensis* (Table 2). Bacteriophage ϕE125 formed plaques on 9 of 10 *B. mallei* strains obtained from NCTC and ATCC. It also formed plaques on DD3008, a capsule-deficient mutant derived from ATCC 23344 (24). Three *B. mallei* strains were resistant to plaque formation by ϕE125, NCTC 120, DB110795 (a laboratory-passaged derivative of ATCC 15310), and BML10 (ATCC 23344 harboring the ϕE125 prophage).

ϕE125 did not form plaques on any of the *B. pseudomallei* or *B. thailandensis* strains used in this study (Table 2). It should be noted that the *B. pseudomallei* strains employed in this study were from a variety of sources; 15 clinical isolates, 30 Thai soil isolates, and 5 Australian soil isolates. Similarly, the *B. thailandensis* strains were isolated in northeastern Thailand (15 strains) and central Thailand (17 strains).

Finally, ϕE125 plaque formation was evaluated with 15 additional species of *Burkholderia*, 4 species of *Pandoraea*, 2 species of *Pseudomonas*, *Ralstonia solanacearum*, *Stenotrophomonas maltophilia*, *S. enterica* serovar Typhimurium, *Serratia marcescens*, and *E. coli*. None of these bacteria formed plaques with bacteriophage ϕE125 (Table 2). These results demonstrate that bacteriophage ϕE125 forms plaques only on *B. mallei* strains, that ϕE125-resistant *B. mallei* strains exist, and that the capsular polysaccharide (24) is not required for plaque formation by ϕE125.

ϕE125 is a new member of the family *Siphoviridae*. Bacteriophage may be tailed, cubic, filamentous, or pleomorphic and can be classified by morphotype and host genus (2). Numerous negatively stained bacteriophage were examined, and a representative image of ϕE125 is shown in Fig. 1. ϕE125 possessed an isometric head of 63 nm in diameter and a long noncontractile tail of 203 nm in length and 8 nm in diameter. Based on its B1 morphotype, ϕE125 can be classified as a member of the order *Caudovirales* and the family *Siphoviridae* (1, 2). To our knowledge, this is the first bacteriophage of the *Siphoviridae* family described as being harbored by the host genus *Burkholderia* (2).

LPS O antigen is required for plaque formation by ϕE125. Of the 10 *B. mallei* strains obtained from NCTC and ATCC, only NCTC 120 was resistant to plaque formation by ϕE125 (Table 2). We hypothesized that resistance was due to the absence of a surface receptor for ϕE125 on NCTC 120. The result obtained with DD3008 demonstrated that the capsular polysaccharide was not the ϕE125 receptor (Table 2). We next performed an immunoblot on whole-cell lysates of the NCTC and ATCC strains with a commercially available monoclonal antibody (3D11) that reacts with *B. mallei* LPS O antigen (Fig. 2A). All of the NCTC and ATCC *B. mallei* strains, with the exception of NCTC 120, demonstrated a typical ladder LPS appearance after immunostaining with 3D11 (Fig. 2A). The laboratory-passaged derivative of ATCC 15310, termed DB110795, also does not form plaques with ϕE125 (Table 2). We performed an immunoblot on a whole cell lysate of DB110795 with the monoclonal antibody 3D11 and found that it did not produce LPS O antigen (Fig. 2B). These results demonstrate that there is a correlation between the absence of LPS O antigen and resistance to plaque formation by ϕE125.

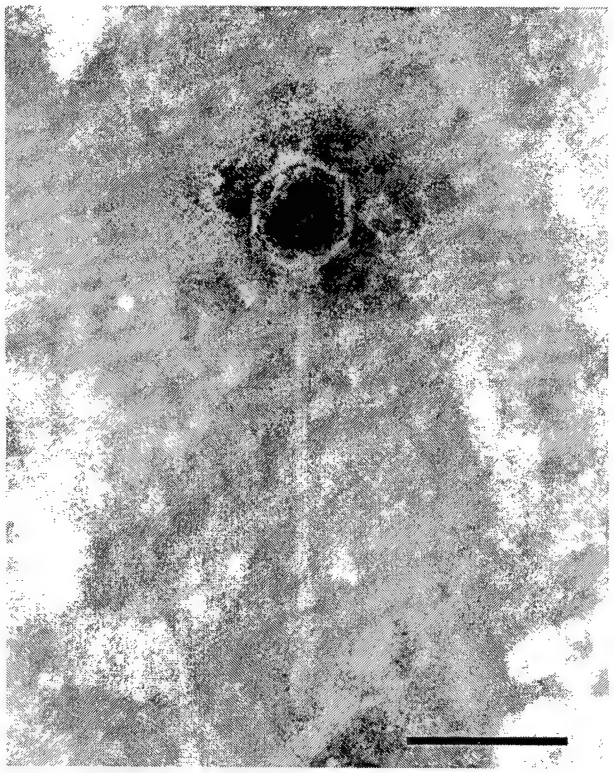

FIG. 1. Transmission electron micrograph of bacteriophage ϕE125 negatively stained with 1% phosphotungstic acid. Scale bar, 100 nm.

A previous study demonstrated that IS*407*A is active in *B. mallei* during serial subculture in vitro. IS*407*A integrated into the capsule gene cluster in *B. mallei* DD420 and resulted in a capsule-deficient strain (24). The LPS O-antigen gene clusters of NCTC 120 and DB110795 were analyzed to determine if this 1.2-kb insertion element (IS) was responsible for the lack of LPS O-antigen production by these strains. The nucleotide sequence of the *B. pseudomallei* LPS O-antigen gene cluster is known (23), and it was used to design eight PCR primer pairs that would result in 2-kb amplicons spanning the LPS O-antigen locus in *B. mallei*. Eight 2-kb amplicons were generated when PCR assays were performed with these primer pairs and genomic DNA from *B. pseudomallei* 1026b and *B. mallei* ATCC 23344 (data not shown). When the PCR assays were performed with genomic DNA from NCTC 120 and DB110795, seven 2-kb amplicons and one 3.2-kb amplicon were produced (data not shown). The 3.2-kb amplicons generated using primer pairs 7-1–7-2 (NCTC 120) and 8-1A–8-2A (DB110795) were cloned and sequenced. The sequencing results demonstrate that NCTC 120 and DB110795 harbor IS*407*A insertions in *wbiE* and *wbiG*, respectively. There was a 4-bp duplication of the sequence 5′-CTGC-3′ flanking the insertion site in NCTC 120 and a 4-bp duplication of the sequence 5′-GCAG-3′ flanking the insertion site in DB110795.

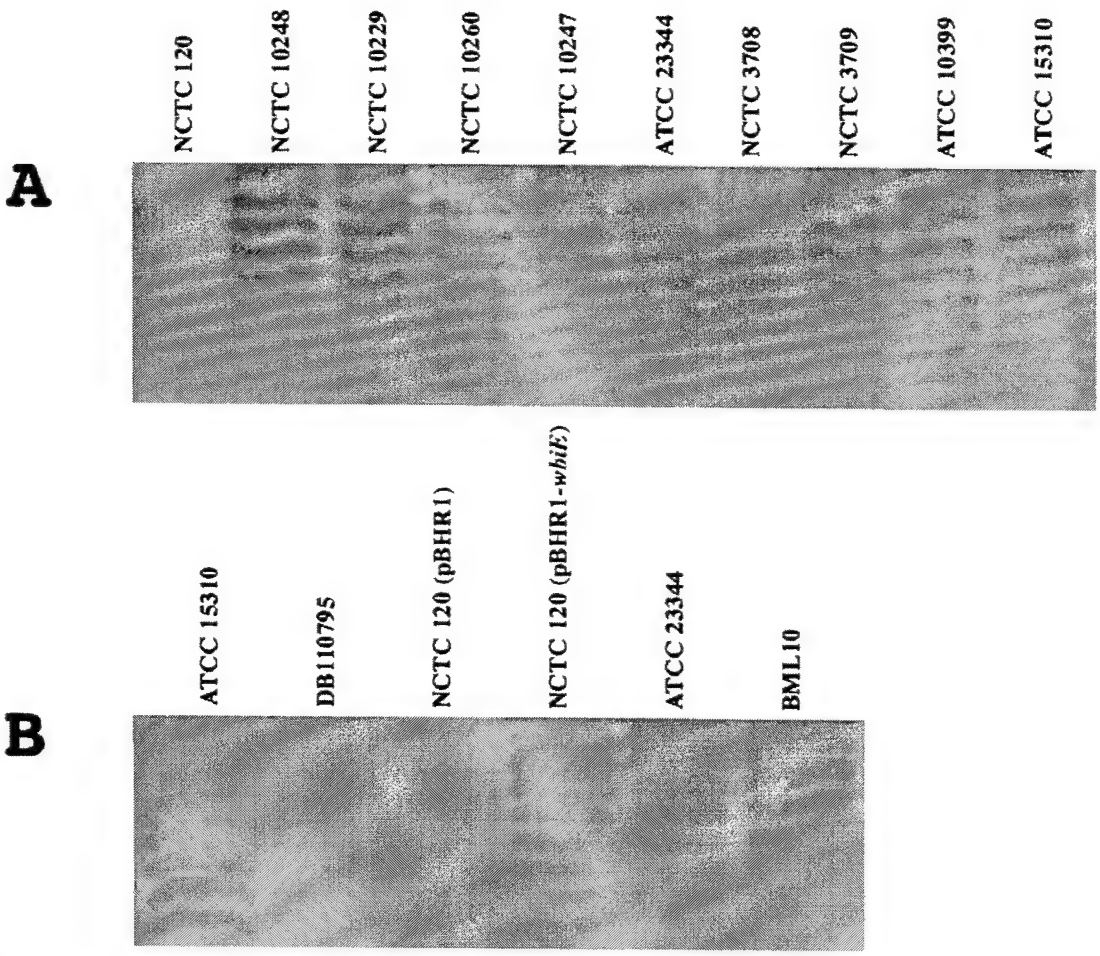

FIG. 2. Immunoblot analysis of *B. mallei* LPS O antigens. Bacteria were washed, resuspended in SDS-PAGE sample buffer, boiled, treated with proteinase K, and subjected to SDS-PAGE. The LPS O antigens were blotted to a polyvinylidene difluoride membrane and reacted with the monoclonal antibody 3D11. (A) LPS O-antigen profiles of NCTC and ATCC *B. mallei* strains. (B) Comparative LPS O-antigen profiles of ϕE125-resistant and ϕE125-susceptible *B. mallei* strains. All strains form plaques with bacteriophage ϕE125 except NCTC 120, DB110795, and NCTC 120 (pBHR1).

Interestingly, the *B. mallei* capsule mutant DD420 harbors an IS*407*A insertion in *wcbF* that is also flanked by a duplication of the sequence 5′-GCAG-3′ (24).

The *wbiE*::IS*407*A mutation in NCTC 120 was complemented by providing the *wbiE* gene from ATCC 23344 in *trans* on the broad-host-range plasmid pBHR1 (Table 1). Figure 2B shows that NCTC 120 (pBHR1) does not produce LPS O antigen but that NCTC 120 (pBHR1-*wbiE*) does. Furthermore, NCTC 120 (pBHR1-*wbiE*) formed plaques with ϕE125, but NCTC 120 (pBHR1) did not. These results demonstrate that the lack of LPS O-antigen production by NCTC 120 is due to an IS*407*A mutation in *wbiE* and that the LPS O antigen is required for plaque formation by ϕE125.

BML10 is immune to ϕE125 superinfection and produces LPS O antigen. Lysogenic bacteria are resistant to superinfection by the temperate bacteriophage that they harbor. Following infection, the ϕE125 genome integrates in the *B. mallei* chromosome at a specific site and becomes a prophage (see below). ATCC 23344 was infected with ϕE125, and a lysogenic derivative was isolated and designated BML10. *B. mallei* BML10 spontaneously produced approximately 500 ϕE125 per ml of broth culture. In comparison, *B. thailandensis* E125 spontaneously produced approximately 1,100 ϕE125 per ml of broth culture. As shown in Table 2, ϕE125 does not form plaques on BML10. Whole-cell lysates of ATCC 23344 and BML10 were analyzed by immunoblot analysis with the monoclonal antibody 3D11, and both strains produced a typical LPS O-antigen banding pattern (Fig. 2B). As shown above, NCTC 120 and DB110795 are resistant to infection with ϕE125 because they do not produce LPS O antigen. BML10, on the other hand, produces LPS O antigen but is still resistant (immune) to ϕE125 superinfection, probably via a prophage-encoded gene product(s). It should be noted that *B. thailandensis* E125 also harbors the ϕE125 prophage and is also immune to superinfection with ϕE125 (Table 2).

Molecular characterization of the bacteriophage ϕE125 genome. The ϕE125 genome was digested with *Hin*dIII, and eight fragments were generated of the following sizes: 1.0, 3.2,

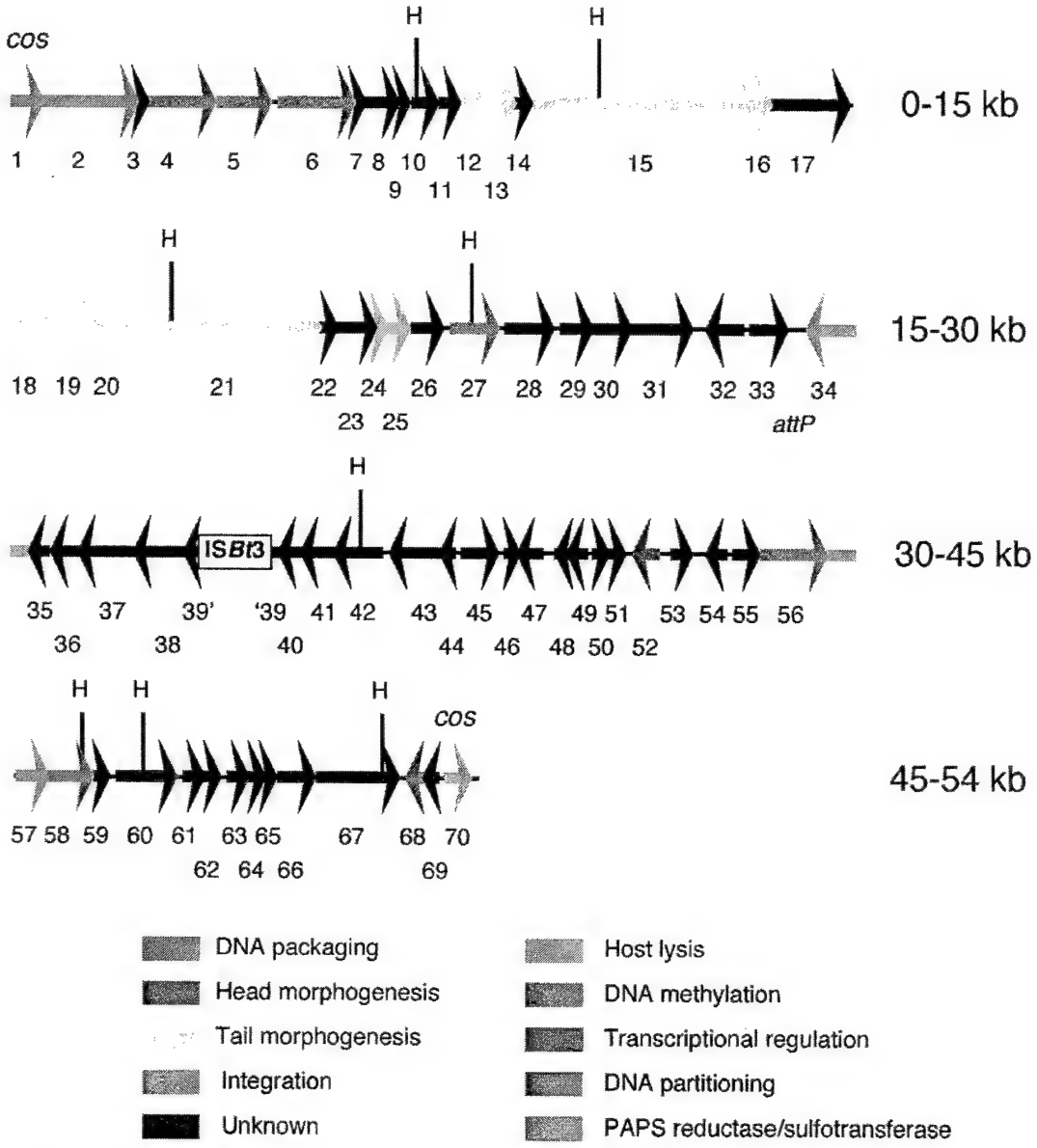

FIG. 3. Physical and genetic map of the bacteriophage φE125 genome. The locations and directions of transcription of genes are represented by arrows, and the gene names are shown below. The locations of *Hin*dIII endonuclease restriction sites are shown (H), and the insertion sequence IS*Bt*3 is represented as a rectangle. The locations of the cohesive (*cos*) and bacteriophage attachment (*attP*) sites are shown above and below the φE125 genome, respectively. The putative functions of proteins encoded by φE125 genes are color coded.

4.4, 5.5, 7.3, 9.0, 9.9, and 13.0 kb. The fragments were heated to 80°C, and the 9.0-kb fragment dissociated into two fragments (1.7 and 7.3 kb), suggesting the presence of a cohesive (*cos*) site on this fragment (data not shown). The eight *Hin*dIII fragments were cloned, and their nucleotide sequences were determined. The nucleotide-sequencing results are depicted schematically in Fig. 3, and pertinent features of φE125 genes and gene products are shown in Table 3.

The φE125 genome is a linear molecule of 53,373 bp in length, and it contains 10-base 3′ single-stranded extensions on the left (3′-GCGGGCGAAG-5′) and right (5′-CGCCCGCTT C-3′), as depicted in Fig. 3. The G + C content of the φE125 genome is 61.2%, which is lower than the 69.3% G + C content of the *B. thailandensis* genome (77). The φE125 genome encodes 70 proteins, and 44% of them show no homology to proteins in the GenBank databases using the BLASTP search algorithm (Table 3 and Fig. 3). The bacteriophage genome also harbors a novel IS*3* family insertion sequence (45), designated IS*Bt*3 (Table 3 and Fig. 3). IS*Bt*3 is 1,318 bp in length, and it has 27-bp terminal inverted repeats flanked by a 3-bp direct duplication. IS*Bt*3 integrated into φE125 gene*39*, suggesting that the encoded protein (gp39) is not essential for a productive lysogenic infection.

Twenty-eight proteins encoded by φE125 are similar to pro-

TABLE 3. Characteristics of bacteriophage ϕE125 genes and gene products

Gene	Orientation[a]	Start (position)	End (position)	Size of protein (kDa)	Protein function and homologs
1	R	46	531	17.2	Terminase (small subunit); phage GMSE-1 Orf16; 1e-12; AF311659; phage 7201 Orf21; 2e-09; AF145054
2	R	541	2253	64.2	Terminase (large subunit); *E. coli* YmfN; 1e-162; NP_415667; phage D3 terminase; 1e-125; NP_061498
3	R	2250	2435	6.3	
4	R	2440	3699	46.4	Portal protein; *H. influenzae* Orf25-like protein; 1e-40; AAF27362; CP-933C Z1849; 1e-40; NP_287334
5	R	3759	4664	31.7	Capsid assembly protein/protease; phage WO Orf7; 8e-44; AB036665; prophage Gifsy-1 STM2605; 2e-34; AE008818
6	R	4767	6074	46.3	Major capsid protein; CP-933N Z1804; 2e-09; NP_287292; CP-933M Z1360; 2e-09; NP_286882
7	R	6134	6319	6.4	
8	R	6326	6892	20.6	
9	R	6892	7218	12.1	Phage HK022 gp9; 3e-08; AF069308
10	R	7211	7633	15.4	Phage HK97 gp10; 4e-20; AF069529 CP-933M Z1368; 1e-19; NP_286890
11	R	7630	7977	12.1	
12	R	8039	8497	16.4	Major tail subunit protein; phage HK97 gp12; 2e-20; NP_037706; *E. coli* ECs1800; 7e-17; NP_309827
13	R	8519	8989	17.4	Tail assembly chaperone protein; phage HK97 gp13; 9e-06; NP_037708; phage HK97 gp14; 1e-05; NP_037707
14	R	8989	9273	10.0	Phage HK97 gp14; 9e-07; NP_037707
15	R	9287	13351	143.0	Tail length tape measure protein; CP-933P Z6034; 2e-25; NP_287971; *E. coli* ECs2240; 2e-25; NP_310267
16	R	13348	13686	12.5	Minor tail protein; phage HK97 gp17; 1e-18; NP_037711; phage HK022 gp17; 3e-18; NP_037677
17	R	13695	15083	50.1	
18	R	15080	15763	25.2	Minor tail protein; *P. aeruginosa* PA0638; 2e-62; G83565; phage N15 gp18; 3e-51; AF064539
19	R	15783	16565	28.6	Tail component protein; *P. aeruginosa* PA0639; 1e-51; AE004499; phage HK97 gp19; 1e-43; AF069529
20	R	16562	17146	20.1	Tail component protein; *Y. pestis* YPO2129; 3e-33; AJ414151; phage N15 gp20; 3e-28; AF064539
21	R	17143	20448	118.3	Tail tip fiber protein; *Y. pestis* YPO2131; < 1e-119; AJ414151; phage N15 gp21; < 1e-119; AF064539
22	R	20445	20759	11.6	
23	R	20759	21493	27.6	
24	R	21536	21748	7.6	Class II holin; phage PS119 gp13; 8e-04; AJ011581; phage PS34 gp13; 8e-04; AJ011580
25	R	21826	22230	14.7	Lysozyme; *X. fastidiosa* XF0513; 2e-13; AE003900; phage PS119 gp19; 2e-11; AJ011581
26	R	22227	22775	18.6	
27	R	22918	23706	30.2	DNA adenine methylase; phage GMSE-1 Orf10; 1e-40; AF311653; *A. lwoffii Alw*I methylase; 5e-19; AF431889
28	R	23815	24693	30.9	
29	R	24875	25435	19.1	*P. aeruginosa* PA1508; 7e-07; AE004579; *Y. pestis* YPO0866; 8e-07; AE004579
30	R	25432	26166	26.3	*P. aeruginosa* PA0822; 5e-23; AE004517; *P. aeruginosa* PA0823; 4e-09; AE004517
31	R	26194	27285	39.4	*P. aeruginosa* PA0821; 1e-48; AE004517
32	L	28059	27418	25.0	
33	R	28145	28816	25.3	Plasmid pFKN Orf11; 2e-17; AF359557; plasmid pNL1 Orf520; 3e-04; AF079317
		29062	29014		*attP* (3′ end of tRNA ProUGG)
34	L	30292	29192	42.2	Site-specific integrase; prophage XfP2 XF2530; 6e-09; AE004060; CP-933M Z1323; 1e-08; NP_286846
35	L	30609	30292	11.8	
36	L	31142	30642	19.3	
37	L	32131	31139	37.2	*M. tuberculosis* Rv2734; 8e-32; NP_217250; *N. punctiforme* hypothetical protein; 4e-28; AAK68643
38	L	33180	32128	37.9	
39′	L	33314	33195		
tnpB	L	34204	33350	32.6	Transposase (IS*3* family); IS*868* ORF4; 1e-101; X55075; IS*401* transposase subunit; 4e-99; L09108
tnpA	L	34539	34261	10.7	Transposase (IS*3* family); IS*401* transposase subunit; 6e-29; L09108; IS*868* Orf1; 9e-20; X55075
′39	L	34794	34636		

Continued on following page

TABLE 3—*Continued*

Gene	Orientation[a]	Start (position)	End (position)	Size of protein (kDa)	Protein function and homologs
40	L	35195	34794	14.6	Phage M×8 p77; 0.1; AF396866
41	L	35863	35192	25.1	CP-9330 Z2097; 4e-05; AE005346; CP-933U Z3120; 2e-04; AE005422
42	L	36671	35877	29.8	
43	L	37670	36858	29.7	*Y. pestis* YPMT1.49c; 1e-38; NC_003134; *S. enterica* HCM2.0006c; 2e-38; AL513384
44	L	37816	37667	5.5	
45	R	38065	38718	28.6	
46	R	38919	39173	9.1	
47	L	39586	39170	15.5	
48	L	39993	39862	4.7	
49	L	40278	40003	10.4	
50	R	40519	40662	5.0	
51	R	40747	40935	7.0	
52	L	41555	41163	14.4	Repressor protein; *X. fastidiosa* XF0499; 9e-14; AE003899; prophage e14 protein b1145; 9e-04; F64859
53	R	42006	42338	11.8	
54	L	42821	42567	9.4	
55	R	43007	43447	15.8	Phage phi CTX Orf33; 0.24; BAA36261
56	R	43452	44588	42.1	DNA cytosine methylase; *C. freundii Cfr*9I methylase; 8e-45; X17022; *P. alcaligenes Pac*25I methylase; 1e-44; U88088
57	R	44585	45583	36.8	PAPS reductase/sulfotransferase; phage 186 Orf84; 6e-55; U32222; *A. pernix* APE2075; 8e-07; F72512
58	R	45618	46439	29.9	DNA partitioning protein; *R. equi* ParA; 1e-06; NP_066815; *L. lactis* ParA; 2e-05; NP_266252
59	R	46436	46696	9.5	
60	R	46850	47842	36.3	CP-933R Z2397; 7e-06; NP_287824
61	R	48035	48388	13.9	Prophage pi3 protein 45; 4e-05; AL596172; *L. monocytogenes* lmo2306; 6e-05; CAD00384
62	R	48385	48741	13.2	
63	R	48843	49181	12.6	
64	R	49172	49516	13.1	
65	R	49492	49752	9.8	
66	R	49761	50408	24.1	
67	R	50482	51876	51.3	*S. meliloti* SMa0594; 6e-11; NP_435557
68	L	52603	52217	14.1	Helix-turn-helix transcriptional regulator; *S. meliloti* SMc00089; 4e-10; NP_385047; *A. tumefaciens* AGR_C_1081p; 2e-04; NP_353634
69	L	52857	52600	9.3	*P. horikoshii* PHS013; 1e-04; NP_142388; *P. jensenii* Orf10; 0.006; CAC38044
70	R	52916	53272	13.1	Class I holin; *X. nematophila* prophage Orf7; 1e-16; CAB58450; *H. influenzae* holin-like protein; 4e-08; AF198256

[a] R, right; L, left.

teins encoded by other bacteriophage, prophage, or prophage-like elements (Table 3). Interestingly, there are numerous similarities to HK022 and HK97 (34) and to λ-like cryptic prophages in *E. coli* O157 Sakai (52) and *E. coli* O157 EDL933 (53). Bacteriophage genomes are composed of a mosaic of multigene modules, each of which encodes a group of proteins involved in a common function, such as DNA packaging, head biosynthesis, tail biosynthesis, host lysis, lysogeny, or replication (11, 28, 34, 37). The ϕE125 genome contains a unique combination of multigene modules involved in DNA packaging, head morphogenesis, tail morphogenesis, and host lysis (Fig. 3 and Table 3). The relative order of these modules in the ϕE125 genome is similar to that of other *Siphoviridae* genomes (11, 34, 37, 42). Since ϕE125 possesses both structural and genetic similarities to the λ supergroup group of *Siphoviridae*, it probably should be included with λ, N15, HK97, HK022, and D3 in the λ-like genus (11).

Early bacteriophage gene functions (lysogeny and replication) are typically located on the right half of *Siphoviriae* genomes, as depicted in Fig. 3 (11). However, the putative lysogeny and replication modules of ϕE125 appear to be unique relative to other members of the *Siphoviridae*. Some of the unusual proteins encoded by the right half of the ϕE125 genome include a DNA adenine methylase (gp27), a DNA cytosine methylase (gp56), a 3′-phosphoadenosine-5′-phosphosulfate (PAPS) reductase or PAPS sulfotransferase (gp57), and a chromosome partitioning protein (gp58) (Fig. 3 and Table 3). The ϕE125 genome also contains two putative holins, gp70 (class I) and gp24 (class II), to coordinate the programmed release of lysozyme (gp25) from the cytoplasm prior to bacteriophage release (72). It is currently unknown if gp70, gp24, or both gp70 and gp24 are required for membrane permeabilization during the ϕE125 life cycle. Finally, several recently sequenced bacterial genomes also encode proteins with similarities to gp29, gp30, gp31, gp33, gp37, gp43, gp61, gp67, gp68, and gp69 (Table 3), suggesting the presence of prophages or

prophage remnants in these bacterial genomes. Alternatively, ϕE125 may have acquired these genes via horizontal transfer from a bacterial host, and they may provide a selective advantage to a lysogen harboring this bacteriophage.

ϕE125 integrates into a proline tRNA (UGG) gene in *B. thailandensis* and *B. mallei*. As with other lambdoid bacteriophages, ϕE125 DNA probably circularizes at the *cos* sites after it is injected into the bacterial cell and follows one of two possible pathways (14). The circularized genome may replicate and produce bacteriophage progeny (lytic response), or it may integrate into the bacterial chromosome and be maintained in a quiescent state (lysogenic response). Temperate bacteriophage genomes often contain an attachment site (*attP*) that they utilize to integrate into a homologous region on the bacterial genome (*attB*) via site-specific recombination (18). Since ϕE125 encodes a site-specific integrase (gp34), we were interested in identifying where the ϕE125 genome was integrated in *B. thailandensis* E125 and *B. mallei* BML10 and in determining the nucleotide sequences of *attP* and *attB*.

Chromosomal DNA flanking one side of the ϕE125 attachment site in *B. thailandensis* E125 was cloned and sequenced (see Materials and Methods). The nucleotide sequence of this region contained a 49-bp sequence that was identical for the ϕE125 genome and the *B. thailandensis* E125 chromosome. This sequence corresponded to the 3′ end of a 77-bp proline tRNA (UGG) gene on the *B. thailandensis* chromosome (Fig. 4A). tRNA genes often serve as target sequences for site-specific integration of temperate bacteriophages, plasmids, and pathogenicity islands (27, 63). Immediately upstream of the proline tRNA (UGG) gene on the *B. thailandensis* chromosome was a divergently transcribed gene designated *orfB* (Fig. 4B). BLASTP results demonstrated that OrfB was 52% identical to RSc1539, a probable hydrolase protein from *R. solanacearum*. The *B. thailandensis* proline tRNA (UGG) gene and *orfB* were also present in the *B. mallei* ATCC 23344 genome (http://www.tigr.org/), and they were 100 and 91% identical at the nucleotide level, respectively. Downstream of the proline tRNA (UGG) gene in *B. mallei* ATCC 23344 was *orfA*, a gene that encoded a protein with 40% identity to RSc2888, a hypothetical protein from *R. solanacearum* (Fig. 4B). In order to determine if ϕE125 integrates in the 3′ end of the tRNA proline (UGG) gene in *B. mallei*, we designed PCR primers specific for *B. mallei orfA* and ϕE125 gene*34* (Fig. 4B). *B. mallei* ATCC 23344 and ϕE125 DNA did not yield a PCR product with these primers, but *B. mallei* BML10 did (data not shown). These results, represented schematically in Fig. 4B, demonstrate that bacteriophage ϕE125 integrates into the 3′ end of the proline tRNA (UGG) gene in *B. mallei* and *B. thailandensis*. It should also be noted that attachment at this site leaves the proline tRNA (UGG) gene intact on the right side, as depicted in Fig. 4B.

Survey of *B. thailandensis* strains for the presence of ϕE125-like prophages. As mentioned above, lysogenic bacteria are immune to superinfection with the same (or similar) bacteriophage that they harbor. The results presented in Table 2 demonstrate that all thirty-two *B. thailandensis* strains in our collection, including E125, are resistant to infection with ϕE125. To determine if the strains were resistant to infection because they harbored ϕE125-like prophages, genomic DNA was isolated from all strains and PCR was performed with primer pairs specific for four distinct regions of the ϕE125 genome. The primer pairs used were 9.5R and 3.2R (gene*9* and gene*10*), 7.5F and 5.5F (gene*21*), 18R and 11F (gene*42*), and 4.4R and 9.5F (gene*67*). Only ten of the thirty-two *B. thailandensis* strains yielded positive PCR results with these primer pairs (E96, E100, E125, E253, E254, E256, E263, E264, E286, and E293). As expected, E125 was positive for all of the PCR primer pairs. The only other strain that was positive for all four primer pairs was E286. Strains E253 and E264 yielded positive PCR results for two primer pairs, and all of the other strains were positive for three primer pairs. All 10 *B. thailandensis* strains spontaneously produced bacteriophage that formed plaques on *B. mallei* ATCC 23344. Thus, it appears that E96, E100, E253, E254, E256, E263, E264, E286, and E293 all harbor ϕE125-like prophage and may be immune to superinfection with ϕE125. On the other hand, 22 *B. thailandensis* strains did not yield a positive PCR product with any of the primer pairs and probably do not harbor a ϕE125-like prophage. These observations suggest that the molecular mechanism of ϕE125 resistance in these strains is probably not due to superinfection immunity.

Functional analysis of the putative DNA methyltransferases of ϕE125. ϕE125 encodes two proteins, gp27 and gp56, that contain similarities to Type II DNA methyltransferases (Table 3). Site-specific DNA methylation usually leads to the formation of three different products: N6-methyladenine (m6A), 5-methylcytosine (m5C), and N4-methylcytosine (m4C). Some tailed bacteriophage genomes contain unusual or modified DNA bases that may be important in protecting the infecting bacteriophage DNA from host restriction endonucleases (1). gp27 is a putative DNA adenine methylase, and gp56 is a putative DNA cytosine methylase. We were interested in determining if gp27 and gp56 were functional DNA methyltransferases.

The plasmids pCR2.1, pAM1, and pCM1 (Table 1) were transformed into *E. coli* DB24, a strain that is deficient in all of the *E. coli* DNA methylases (36), and DNA methylase dot blot assays were performed with rabbit primary antibodies specific for m6A and m4C. Figure 5A shows that the m6A antibody reacted with genomic DNA samples from λ (positive control) and DB24 (pAM1) but did not react with DB24 (pCR2.1) or DB24 (pCM1). The m6A antibody also reacted with genomic DNA samples from *B. mallei* BML10 and bacteriophage ϕE125 (Fig. 5A). On the other hand, there was only background reactivity of the m6A antibody with genomic DNA from *B. thailandensis* E125 and *B. mallei* ATCC 23344 (Fig. 5A). It appears that the ϕE125 m6A methylase has little or no activity in the *B. thailandensis* lysogen but is very active in the *B. mallei* lysogen (Fig. 5A, compare E125 and BML10). It is currently unclear if the *B. mallei* BML10 genome contains m6A or if the positive signal obtained with the m6A antibody is due to the ϕE125 genome, which also contains m6A (Fig. 5A). Taken together, these results clearly demonstrate that gene*27* is expressed in DB24, that gp27 is a functional m6A methylase, and that the ϕE125 genome contains m6A.

The m4C antibody did not react with genomic DNA from DB24 (pCR2.1), DB24 (pAM1), DB24 (pCM1), or *B. thailandensis* E125, but it did react with DB24 genomic DNA methylated with M.*Rsa*I as a positive control (Fig. 5B). This indicates that gene*56* is not expressed or is inactive in DB24 (pCM1) and *B. thailandensis* E125. On the other hand, positive

A

5′ CGGAGCGTAGCGCAGCCTGGTAGCGCATCTGATT**TGG**GAT

CAGAGGGTCGTAGGTTCGAATCCTATCGCTCCGACCA 3′

B

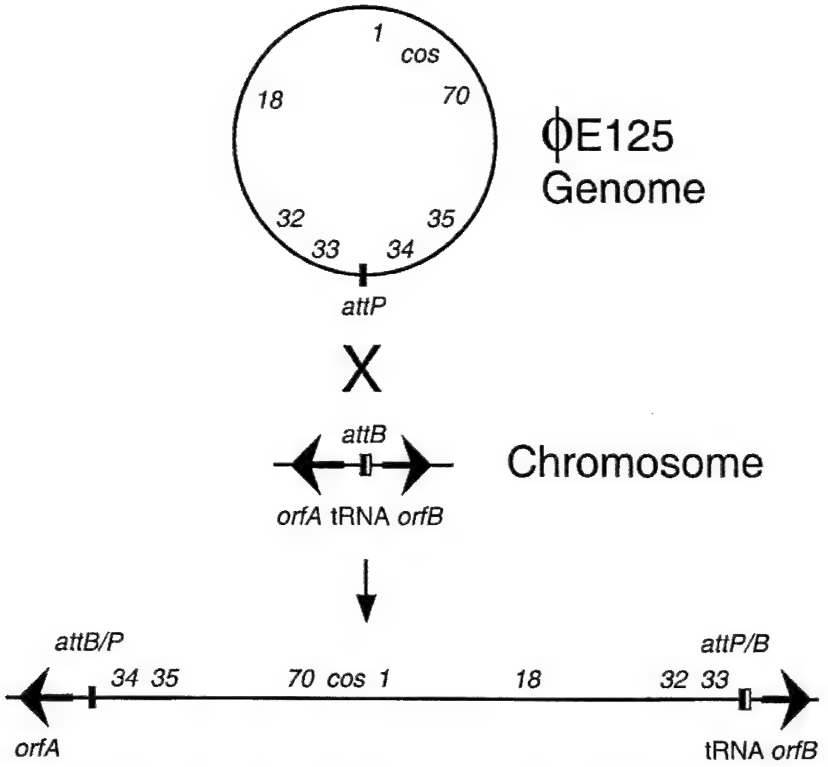

FIG. 4. Bacteriophage ϕE125 integrates into the proline tRNA (UGG) gene in *B. mallei* and *B. thailandensis*. (A) The nucleotide sequence of the proline tRNA (UGG) gene of *B. mallei* ATCC 23344 and *B. thailandensis* E125. The underlined sequence represents the 49-bp attachment site that is identical in the ϕE125 genome (*attP*), the *B. mallei* chromosome (*attB*), and the *B. thailandensis* chromosome (*attB*). The location of the anticodon in the proline tRNA gene is shown in bold. (B) Schematic representation of integration of the ϕE125 genome into the proline tRNA (UGG) gene of *B. mallei* and *B. thailandensis*. The ϕE125 genome is depicted as a circle, and the approximate locations of gene*1*, gene*18*, gene*32*, gene*33*, gene*34*, gene*35*, gene*70*, and the *cos* site are shown. The *B. mallei* and *B. thailandensis* chromosomes are represented as a line, and the location and direction of transcription of *orfA* and *orfB* are represented by arrows. The 5′ end of the proline tRNA (UGG) gene is shown as a thin white rectangle, and the 3′ end (the attachment site) is shown as a thin black rectangle. Following site-specific recombination (X), the *orfA* and *orfB* genes are separated by the integrated ϕE125 prophage.

signals were obtained when the m4C antibody was reacted with genomic DNA from *B. mallei* BML10 and ϕE125 (Fig. 5B). It is likely that gp56 is an m4C methylase because genomic DNA from *B. mallei* BML10 reacts with the m4C antibody, but *B. mallei* ATCC 23344 genomic DNA does not (Fig. 5B). Alternatively, ϕE125 infection may activate a cryptic *B. mallei* m4C methylase or a ϕE125 protein other than gp56 may be responsible for the m4C methylase activity in *B. mallei* BML10. It is not clear if the *B. mallei* BML10 genome contains m4C or if the positive signal obtained with the m4C antibody is strictly due to m4C methylation of the ϕE125 genome (Fig. 5B). Further studies will be required to determine the DNA specificities of gp27 and gp56.

DISCUSSION

In this study, we isolated and characterized ϕE125, a tailed bacteriophage specific for *B. mallei*. The host range of ϕE125 was examined by using bacteria from three genera of β-*Proteobacteria* (*Burkholderia*, *Pandoraea*, and *Ralstonia*) and five

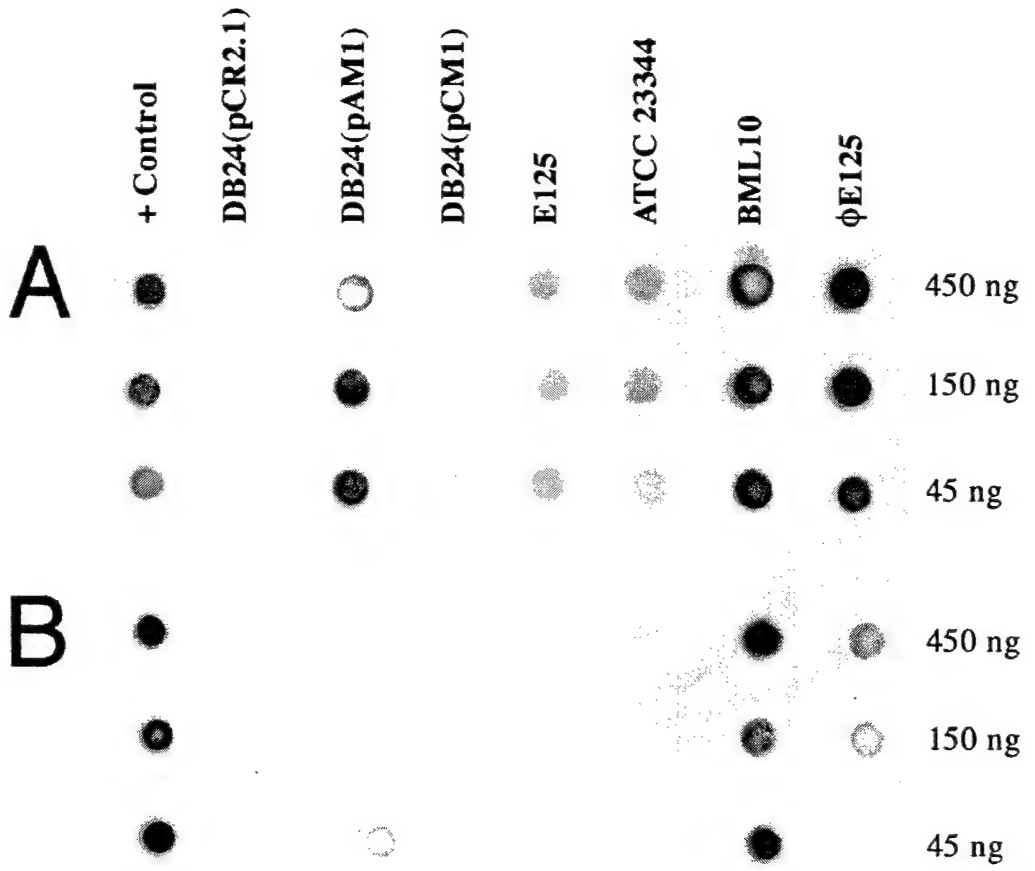

FIG. 5. Dot blot assay to detect genomic DNA methylation using rabbit primary antibodies specific for m6A or m4C. (A) Methylase dot blot assay using polyclonal antibodies specific for m6A. Bacteriophage 2 genomic DNA was used as a positive (+) control. (B) Methylase dot blot assay using polyclonal antibodies specific for m4C. *E. coli* DB24 genomic DNA methylated by M.*Rsa*I served as a positive (+) control. The quantities of genomic DNAs spotted on each panel are shown.

genera of γ-*Proteobacteria* (*Pseudomonas*, *Stenotrophomonas*, *Salmonella*, *Serratia*, and *Escherichia*). In fact, eighteen different *Burkholderia* species were tested, and only *B. mallei* strains were sensitive to φE125 (Table 2). The most-impressive host specificity results were obtained with *B. pseudomallei* and *B. thailandensis*, two species closely related to *B. mallei*. Bacteriophage φE125 did not form plaques on any of the 50 strains of *B. pseudomallei* or 32 strains of *B. thailandensis* tested in this study. Glanders was eradicated from North America in the 1930s and we were able to test only 13 strains of *B. mallei* due to the difficulty of obtaining unique isolates of this species. Nonetheless, the results clearly demonstrate that φE125 specifically forms plaques on *B. mallei*, and we hope to use it, in conjunction with other methods, as a diagnostic tool for *B. mallei*.

The LPS O antigen was required for infection with φE125, suggesting that this molecule is the bacteriophage receptor. This is similar to the λ-like bacteriophage D3, which utilizes the LPS O antigen of *P. aeruginosa* for infection (37, 38). It is surprising that φE125 did not infect *B. pseudomallei* or *B. thailandensis* because the chemical structure of the *B. mallei* LPS O antigen, a heteropolymer of repeating D-glucose and L-talose, is similar to that previously described for these closely related species (10, 12, 35, 54). In fact, the gene clusters encoding the *B. mallei* and *B. pseudomallei* LPS O antigens are 99% identical at the nucleotide level (12, 23). However, unlike *B. pseudomallei* and *B. thailandensis*, the *B. mallei* LPS O antigen is devoid of an *O*-acetyl group at the 4′ position of the L-talose residue. The chemical structure of the *B. mallei* LPS O antigen is as follows: (3)-β-D-glucopyranose-(1,3)-6-deoxy-α-L-talopyranose-(1-, in which the talose residue contains 2-*O*-methyl or 2-*O*-acetyl substituents (12). Our present hypothesis is that *B. pseudomallei* and *B. thailandensis* are resistant to infection with φE125 because the *O*-acetyl group at the 4′ position of the L-talose residue alters the conformation of the LPS O antigen and/or blocks the bacteriophage binding site. *B. pseudomallei* and *B. thailandensis* possess an *O*-acetyltransferase that is responsible for transferring the *O*-acetyl group to the 4′ position of the L-talose residue. This *O*-acetyltransferase gene is not present, is not expressed, or is mutated in *B. mallei*. We are currently attempting to identify the *B. pseudomallei* *O*-acetyltransferase gene and provide it in *trans* to *B. mallei* to see if it *O*-acetylates the 4′ position of L-talose and confers resistance to φE125. Alternatively, inactivation of the *O*-acetyltransferase gene should make *B. pseudomallei* sensitive to φE125.

It is also possible that *B. pseudomallei* and *B. thailandensis* are immune to superinfection with ϕE125 because they harbor a ϕE125-like prophage. The nucleotide sequence of a 1,068-bp *Hin*dIII fragment from a *B. mallei*-specific bacteriophage produced by *B. pseudomallei* 1026b (ϕ1026b) was recently obtained and was found to be 98% identical to the 1,068-bp *Hin*dIII fragment from ϕE125 (D. DeShazer, unpublished data). However, the nucleotide sequences of other *Hin*dIII fragments from ϕ1026b displayed no similarities to ϕE125, indicating that ϕ1026b and ϕE125 are distinct bacteriophages that share regions (modules) of genetic similarity. We found that 10 of the 32 *B. thailandensis* strains in our collection harbor a ϕE125-like prophage, and the genomic sequence of *B. pseudomallei* K96243 also contains several genes that are nearly identical to ϕE125 genes (http://www.sanger.ac.uk/). Thus, it is clear that some *B. pseudomallei* and *B. thailandensis* strains are lysogenic for a ϕE125-like bacteriophage and may be immune to superinfection with ϕE125. It is also important to note that 22 *B. thailandensis* strains in our collection did not possess an ϕE125-like prophage, suggesting that superinfection immunity alone is not responsible for their resistance to infection with ϕE125.

In this study, we found that *B. mallei* NCTC 120 and *B. mallei* DB110795 do not produce LPS O antigens due to IS*407*A insertions in *wbiE* and *wbiG*, respectively. Burtnick et al. (12) have recently obtained identical results with *B. mallei* NCTC 120 and *B. mallei* ATCC 15310, the parental strain of *B. mallei* DB110795. We found that *B. mallei* ATCC 15310 does produce LPS O antigen (Fig. 2A) and does not contain the *wbiG*::IS*407*A mutation. In fact, the ATCC stock cultures (1964 and 1974) of *B. mallei* ATCC 15310 do not harbor IS*407*A insertions in *wbiG* (Jason Bannan, personal communication). *B. mallei* DB110795 was obtained by routine laboratory passage of *B. mallei* ATCC 15310 at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID). The strain used in the study of Burtnick et al. (12) was obtained from USAMRIID and was probably *B. mallei* DB110795, not *B. mallei* ATCC 15310. It was previously reported by members of our group that IS*407*A integrated into a capsular polysaccharide gene during repeated laboratory passage of *B. mallei* ATCC 23344 (24). Taken together, these results suggest that IS*407*A transposition may be relatively common during routine laboratory passage of this microorganism. Serial subculture of *B. mallei* on laboratory media results in a loss of virulence for animals (48, 49, 51, 57), and it is tempting to speculate that IS*407*A transposition is responsible, directly or indirectly, for this phenomenon.

Finally, we found that ϕE125 genomic DNA contained the methylated bases m6A and m4C (Fig. 5). DNA methylation may protect ϕE125 DNA from host restriction endonucleases (1), or it may be involved in some other aspect of the ϕE125 life cycle. We cloned and expressed ϕE125 gene*27* in *E. coli* and found that gp27 was a functional m6A methylase. We were unable to provide direct evidence that gp56 was a m4C methylase, but it was intriguing that ϕE125 DNA and genomic DNA from a *B. mallei* lysogen contained m4C. It was surprising that genomic DNA from a *B. mallei* lysogen contained m6A and m4C, but genomic DNA from a *B. thailandensis* lysogen did not. We are currently examining the possibility that gp27 and gp56 require host factors for production and/or activity that are present in *B. mallei* but not in *B. thailandensis*. Type II DNA methylases specifically bind and methylate recognition sequences on a DNA substrate (58). The DNA sequence specificities of gp27 and gp56 are currently unknown, but BLASTP results show that gp56 is similar to cytosine methylases that recognize and methylate the sequence 5′-CCCGGG-3′, which occurs nine times in the ϕE125 genome. ϕE125 DNA was treated with five restriction endonucleases that recognize this sequence (*Sma*I, *Xma*I, *Cfr*9I, *Psp*AI, and *Xma*CI), and they all cleaved the DNA into nine fragments of the predicted sizes (D. DeShazer and J. A. Jeddeloh, unpublished data). The fact that cleavage was not blocked strongly suggests that this site is not methylated. Further studies are required to determine the specificity of gp27 and gp56 and to understand their role(s) in the ϕE125 life cycle.

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APPENDIX 3

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The *wbiA* locus is required for the 2-*O*-acetylation of lipopolysaccharides expressed by *Burkholderia pseudomallei* and *Burkholderia thailandensis*

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Abstract

Burkholderia pseudomallei and *Burkholderia thailandensis* express similar O-antigens (O-PS II) in which their 6-deoxy-α-L-talopyranosyl (L-6dTal*p*) residues are variably substituted with *O*-acetyl groups at the *O*-2 or *O*-4 positions. In previous studies we demonstrated that the protective monoclonal antibody, Pp-PS-W, reacted with O-PS II expressed by wild-type *B. pseudomallei* strains but not by a *B. pseudomallei wbiA* null mutant. In the present study we demonstrate that WbiA activity is required for the acetylation of the L-6dTal*p* residues at the *O*-2 position and that structural modification of O-PS II molecules at this site is critical for recognition by Pp-PS-W.

Keywords: *Burkholderia* species; O-antigen; Virulence determinant; *trans*-Acylase

1. Introduction

Burkholderia pseudomallei, the etiologic agent of melioidosis, is a Gram-negative bacterial pathogen responsible for disease in both humans and animals [1,2]. Previous studies have demonstrated that the lipopolysaccharide (LPS) expressed by *B. pseudomallei* is both a virulence determinant and a protective antigen [3–6]. Consequently, the O-antigen (O-PS II) has become a significant component of the various sub-unit vaccine candidates that we are currently developing for immunization against melioidosis [7].

The O-PS II moiety produced by *B. pseudomallei* is an unbranched heteropolymer consisting of disaccharide repeats having the structure 3)-β-D-glucopyranose-(1→3)-6-deoxy-α-L-talopyranose-(1→ in which ~33% of the 6-deoxy-α-L-talopyranose (L-6dTal*p*) residues possess 2-*O*-methyl and 4-*O*-acetyl substitutions while the remainder of the L-6dTal*p* residues bear only 2-*O*-acetyl modifications [8,9]. Studies have also demonstrated that the non-pathogenic species *Burkholderia thailandensis* synthesizes an O-antigen with the same repeating unit [10]. Recently, we demonstrated that the O-antigen (O-PS) expressed by *Burkholderia mallei*, the causative agent of glanders, is virtually identical to O-PS II except that it lacks acetyl modifications at the *O*-4 position of the L-6dTal*p* residues [11]. Curiously, however, pairwise comparisons between the *B. mallei* and *B. pseudomallei* O-polysaccharide biosynthetic clusters failed to reveal any sequence differences that could account for the structural dissimilarities observed between O-PS and O-PS II [5,11].

In the current study, we used a combination of molecular and physical approaches to further characterize the role of the *wbiA* locus which is thought to be involved in the acetylation of O-PS II antigens [5].

2. Materials and methods

2.1. Strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli*, *B. pseudomallei* and *B. thailandensis* strains were grown at 37°C in Luria–Bertani (LB) broth or on LB agar. *B. mallei* strains were grown at 37°C in LB broth containing 4% glycerol or on LB agar containing 4% glycerol. For *E. coli*, antibiotics

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were used at the following concentrations: ampicillin (Ap) 100 μg ml^{-1}, gentamicin (Gm) 15 μg ml^{-1} and kanamycin (Km) 25 μg ml^{-1}. For *B. pseudomallei* and *B. thailandensis*, antibiotics were used at the following concentrations: Gm 25 μg ml^{-1}, streptomycin (Sm) 100 μg ml^{-1} and trimethoprim (Tp) 100 μg ml^{-1}. Bacterial strains were maintained at −70°C in 20% glycerol suspensions.

2.2. DNA manipulations and transformations

Molecular and cloning techniques were performed essentially as described by Sambrook et al. [12]. Plasmids were purified using QIAprep spin plasmid minipreps (Qiagen). Genomic DNA was isolated using the Wizard® Genomic DNA Isolation kit (Promega). Competent *E. coli* were transformed using standard methods.

2.3. PCR amplification and sequence analysis of wbiA genes

The *wbiA* genes from *B. thailandensis* ATCC 700388 and *B. pseudomallei* 1026b were PCR amplified from purified chromosomal DNA samples using the wbiA-5′ (5′-GCTCTAGACATGAGATCGTGCTTGAGCG-3′) and wbiA-3′ (5′-GGGGTACCGATAAAGCCAGCCCCACCGG-3′) primer pair; the *Xba*I and *Kpn*I sites in the linker regions are underlined. The primers were designed at the 3′-end of *wzt* and the 5′-end of *wbiB* using the previously described *B. pseudomallei* O-PS II biosynthetic gene cluster (GenBank database accession number AF064070). Reactions were performed using *Taq* polymerase (Invitrogen) as per manufacturer's instructions except that the denaturing temperature was increased to 97°C to compensate for the high G/C content of the chromosomal DNAs. The resulting PCR products were then cloned into pCR2.1-TOPO and sequenced on both strands. Sequence analyses were conducted with the aid of DNASIS version 2.5 (Hitachi) as well as the BLASTX and BLASTP programs [13]. The *Shigella flexneri* bacteriophage SF6 *oac* GenBank accession number is X56800. The *B. thailandensis* nucleotide sequence reported in this study was entered into the GenBank database under accession number AY028370.

2.4. Construction and complementation of wbiA mutants

B. pseudomallei PB604, a strain harboring an insertionally inactivated *wbiA* gene, was previously constructed by DeShazer et al. [5]. The *wbiA* gene of *B. thailandensis* was insertionally inactivated using the allelic exchange vector pPB604Tp resulting in strain BT604. Allelic exchange was performed as previously described [5,14]. Mutants were complemented *in trans* using the broad host range vector pUCP31T harboring a wild-type copy of the *B. pseudomallei wbiA* locus. Plasmids were conjugated to *B. pseudomallei* and *B. thailandensis* as previously described [15].

2.5. Western blot and silver stain analysis

Whole cell lysates were prepared as previously described [16] and used in both Western immunoblot and silver stain analyses. Overnight bacterial cultures were pelleted, resuspended in lysis buffer and boiled prior to SDS–PAGE analysis on 12% gels. Immunoblots were performed as previously described [17] using rabbit polyclonal antisera specific for *B. pseudomallei* O-PS II. Silver stain analyses were performed as previously described [18].

2.6. Purification of LPS and O-PS

LPS was purified using a previously described hot aqueous phenol extraction protocol [7,9]. Delipidation of the LPS molecules was achieved via mild acid hydrolysis (2% acetic acid) followed by size exclusion chromatography (Sephadex G-50) as previously described by Perry et al. [9]. Carbohydrate positive fractions were detected using a phenol–sulfuric acid assay [19]. The purity of the carbohydrate preparations was determined to be >90% in all instances. Protein contamination was determined using bicinchoninic acid assays (Pierce) while nucleic acid contamination was estimated from $OD_{260/280}$ measurements.

2.7. Nuclear magnetic resonance (NMR) spectroscopy analysis

^{13}C-NMR spectra were recorded at 100.5 MHz and the chemical shifts were recorded in ppm relative to an internal acetone standard (31.07 ppm [^{13}C]; Spectral Data Services, Champaign, IL, USA).

3. Results and discussion

3.1. Comparison of wbiA alleles from B. thailandensis and B. pseudomallei

The *wbiA* allele from *B. thailandensis* ATCC 700388 was cloned and sequenced as described in Section 2. Analysis of the 1239-bp open reading frame contained within the cloned PCR product demonstrated sequence identities of 93.6% at the nucleotide and 95.0% at the amino acid levels in comparison to the previously characterized *B. pseudomallei* 1026b *wbiA* allele (Fig. 1). Based upon these preliminary results we predicted that the function of WbiA would be similar in both *B. pseudomallei* and *B. thailandensis*.

Further analysis of the *wbiA* gene products expressed by the two *Burkholderia* species demonstrated the presence of conserved amino acid motifs that defines a family of inner membrane *trans*-acylases. The structural and functional significance of these motifs, however, has yet to be determined. The family includes *Salmonella typhimurium* OafA, *Shigella flexneri* bacteriophage SF6 Oac, *Rhizobium meli-*

Table 1
Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristic(s)	Reference or source
Strains		
E. coli		
SM10	Mobilizing strain: expresses RP4 *tra* genes; Km^r Sm^s	[21]
TOP10	High efficiency transformation	Invitrogen
B. pseudomallei		
1026b	Clinical isolate: Gm^r Km^r Sm^r Pm^r Tp^s	[5]
DD503	1026b derivative: Δ(*amrR-oprA*) *rpsL*; Sm^r Pm^r Gm^s Km^s Tp^s	[5]
PB604	DD503 derivative: *wbiA*::*dhfrIIb*-p15A oriV; Tp^r	[5]
PB605	PB604 (pUCP31T); Gm^r Tp^r	This study
PB606	PB604 (p31wbiA); Gm^r Tp^r	This study
B. thailandensis		
ATCC 700338	Type strain (soil isolate): Gm^r Km^r Sm^r Pm^r Tp^s	[10]
DW503	ATCC 700338 derivative: *rpsL*; Sm^r Pm^r Gm^s Km^s Tp^s	[22]
BT604	DW503 derivative: *wbiA*::*dhfrIIb*-p15A oriV; Tp^r	This study
BT605	BT604 (pUCP31T); Gm^r Tp^r	This study
BT606	BT604 (p31wbiA); Gm^r Tp^r	This study
B. mallei		
ATCC 23344	Type strain (human isolate)	USAMRIID[a]
Plasmids		
pCR2.1-TOPO	TA cloning vector: ColE1 ori; Ap^r Km^r	Invitrogen
pUCP31T	Broad host range vector: OriT pRO1600 ori; Gm^r	[23]
p31wbiA	1.37-kb *B. pseudomallei wbiA* PCR product cloned into the *XbaI*/*KpnI* sites of pUCP31T; Gm^r	This study

[a]United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, USA.

loti ExoZ and *Legionella pneumophila* Lag1 [20]. Interestingly, all are involved in the acetylation of bacterial polysaccharides [20]. A gapped sequence alignment of the WbiA homologues with the Oac *trans*-acylase revealed overall sequence identities of approximately 30% (Fig. 1), a result that is consistent with the family in general.

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Bp    1   MKSEIPVTVP DRFDAADVLG APQSARVADT STLANPADRL STHDNGFGLL  50
Bt    1   MKSEIPVTVP ERFNTADVLG GAQPTRVADR STLANPADRL STHDNGFGLL  50
Sf    1   ---------- ---------- ---------- ---------- MHKSNCFDTA  10

                                                    *   **  ** *
Bp   51   RLLFATMVLW DHAFPLGGFG ADPMWRLTLN QDSMGGICVS GFFAISGFLI  100
Bt   51   RLLFATMVLW DHAFPLGGFG ADPMWRLTLN QDSMGGICVS GFFAISGFLI  100
Sf   11   RLVAAMMVLV SHHYALSGQP EP----YLFG FESAGGIAVI IFFSISGYLI   56

               *   *  *
Bp  101  AKSGMRAD-A LQFAWRRCVR IFPAYWAVLI VTALGVGPII HYVQAGTLHG  149
Bt  101  AKSGMRAD-A LQFAWRRCVR IFPAYWAVLI VTALGVGPII HYVQAGTLHG  149
Sf   57  SKSAIRSDSF IDFMAKRARR IFPALVPCSI LTYFLFGWIL NDFSAEYFS-  105

                                                        *
Bp  150  YWNAALGGPL GYIFNNWRLT IGQYGINDLL RDTTPYGHSI SESVFNGSIW  199
Bt  150  YWNAALGGPL GYIVNNWRLT IGQYGINDLL RDTTPYGRSI SESVFNGSIW  199
Sf  105  ---------- ---HDIVRKT ISSIFMSQAP DADITS--HL IHAGINGSLW  140

          *  *  *
Bp  200  TLIYEAKCYV MVGLFAMFGL LTAHRKVLLA VTVVAWFVLA YQTINPAFSA  249
Bt  200  TLIYEAKCYV LVGLFAVFGL LTEHRPVLLA VTGVSWLVLA VQTINPAFSA  249
Sf  141  TLPLEFLCYI ITG-VAVAHL KNGKAFIVIL LVFVSLSLIG SVSENRDVMF  189

Bp  250  QLVPWAGDRH LVQYGTIFLI GSSAAAYSKS LPISDKLGAF AVVVYLISLF  299
Bt  250  QLVPWAGDRH LVQYGTIFLI GSSAAAYSKS LPISDKLGAF AVVIYLISLF  299
Sf  190  SIPLWLYP-- --LRGLAFFF GATMAMYEKS WNVSNVKITV VSLLAMYAYA  235

Bp  300  KGG----YLL LGYPAMVYAI LWLACRLPRW ARRIGSRNDY SYGIYVFGFL  345
Bt  300  KGG----YLL LGYPAMVYAI LWLACRLPRW ARRIGSRNDY SYGIYVFGFL  345
Sf  236  SYGKGIDYTM TCYILVSFST IAICTSVG-- DPLVKGRFDY SYGVYIYAFP  283

Bp  346  VQQVLAYVGA YKYGFVFYLA ASVFFTFICA WFSWHLIEKR ALALKDWGPG  395
Bt  346  VQQVLAYVGA YKYGFVFYLA ASVFFTFICA WFSWHLIEKR ALALKDWGPG  395
Sf  284  VQQVVINT-- LHMGFYPSML LSAVTVLFLS HLSWNLVEKR FLTRSSP---  328

Bp  396  QGWKYCLARL TMKKEGV 412
Bt  396  QGWKYCLARL TMKKEGV 412
Sf  328  --------KL SLD---- 333
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Fig. 1. Amino acid alignment of *B. pseudomallei* 1026b (Bp) *wbiA*, *B. thailandensis* ATCC 700388 (Bt) *wbiA* and *S. flexneri* phage SF6 (Sf) *oac* gene products. Shaded residues represent identity amongst the aligned sequences. Dots indicate dissimilarities between the Bp and Bt proteins. Asterisks indicate residues conserved amongst members of the family of integral membrane proteins involved in the acylation of exported carbohydrates.

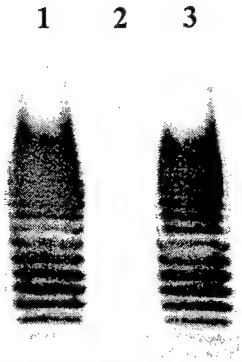

Fig. 2. Western immunoblot analysis of purified *B. thailandensis* LPS antigens. The primary antibody used was the O-PS II specific Pp-PS-W mAb. Lane 1, DW503 LPS; lane 2, BT605 LPS; lane 3, BT606 LPS.

3.2. Phenotypic characterization of wbiA null mutants

To determine the effect of the *wbiA* null mutations on the synthesis of O-PS II, *B. pseudomallei* PB604 and *B. thailandensis* BT604 were phenotypically characterized using a variety of genetic and immunological approaches. Silver staining of SDS–PAGE fractionated whole cell lysates demonstrated that BT604 was capable of expressing full-length LPS molecules based upon the presence of a characteristic LPS banding pattern (data not shown). The LPS was also shown to be immunologically similar to that expressed by the type strain and DW503 due to the reactivity of the antigen with the O-PS II polyclonal antiserum (data not shown). Interestingly, however, neither the BT604 whole cell lysates nor the purified LPS molecules reacted with the O-PS II specific monoclonal antibody (mAb) Pp-PS-W suggesting that the *wbiA* locus was required for the expression of a native O-PS II moieties (Fig. 2). By complementing BT604 with the broad host range vector, p31wbiA, we were able to restore the reactivity of the whole cell lysates and purified LPS with the Pp-PS-W mAb (Fig. 2). Similar results were observed for the *B. pseudomallei* strains (data not shown).

3.3. Spectroscopic analysis of the O-polysaccharide antigens

The O-polysaccharides from *B. thailandensis* DW503, BT604 and BT606, *B. pseudomallei* DD503, PB604 and PB606 and *B. mallei* ATCC 23344 were isolated and purified as described in Section 2. The ^{13}C-NMR spectrum of the DW503 antigen demonstrated four anomeric carbon signals between 98.5 and 102.6 ppm, two *O*-acetyl signals at 174.1 and 174.6 ppm (CH_3CO) as well as 21.2 and 21.4 ppm (CH_3CO), two 6-deoxyhexose CH_3 signals at 16.0 and 16.2 ppm and an *O*-methyl signal at 58.8 ppm (Fig. 3A), all of which are consistent with previously published values [9]. Similar spectra were also obtained for the

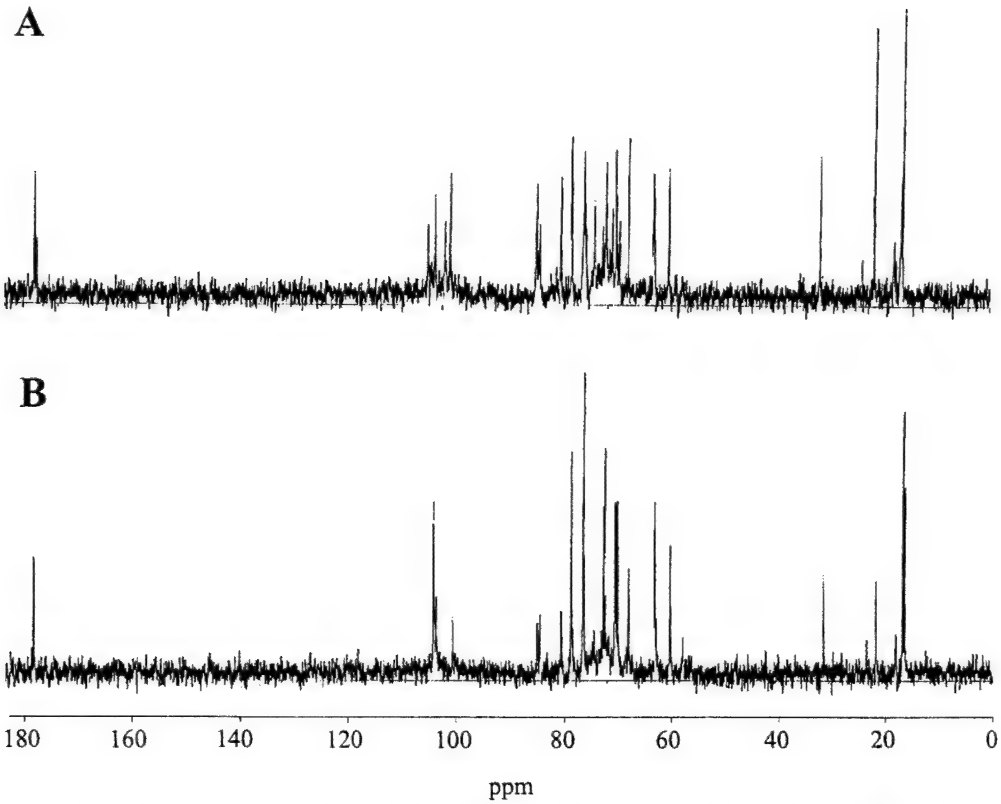

Fig. 3. ^{13}C-NMR spectra of native and mutant O-polysaccharides expressed by *B. thailandensis* strains (A) DW503 and (B) BT604.

BT606, DD503 and PB606 samples (data not shown). In contrast, the ^{13}C-NMR spectrum of the BT604 sample demonstrated four anomeric carbon signals between 98.5 and 102.2 ppm, one *O*-acetyl signal at 174.6 ppm (CH_3CO) and 21.2 (CH_3CO), two 6-deoxyhexose CH_3 signals at 16.0 and 16.3 ppm and an *O*-methyl signal at 58.8 ppm (Fig. 3B). A similar spectrum was recorded for the PB604 sample (data not shown). Based upon these results it was apparent that the O-polysaccharides expressed by BT604 and PB604 were lacking one of the two *O*-acetyl moieties associated with native O-PS II molecules.

To determine which of the *O*-acetyl groups was missing a comparison of the DW503 and BT604 ^{13}C-NMR spectra with the ^{13}C-NMR spectrum obtained for *B. mallei* ATCC 23344 O-PS was conducted. Based upon an analysis of the spectral data we were able to establish that BT604 lacks *O*-acetyl modifications at the *O*-2 position of the L-6dTal*p* residues since O-polysaccharides lacking *O*-acetyl substitutions only at the *O*-4 position would have produced spectra consistent with that obtained for *B. mallei* O-PS (Fig. 4). Similar conclusions can also be drawn for *B. pseudomallei* PB604. Based upon these observations, it is highly probable that a second unlinked locus is responsible for the *O*-acetylation of L-6dTal*p* residues at the *O*-4 position since the *wbiA* locus is the only predicted *trans*-acylase in the O-PS II biosynthetic operon. Studies are currently under way to examine this hypothesis.

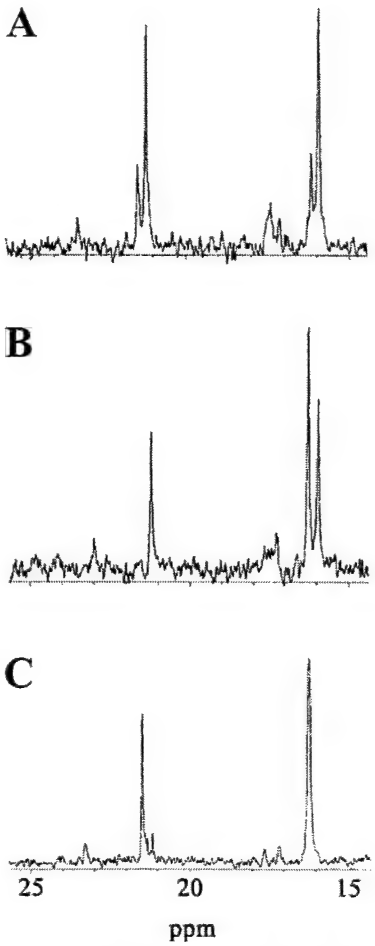

Fig. 4. ^{13}C-NMR spectra of *B. thailandensis* and *B. mallei* O-polysaccharides expanded between the region of 15–25 ppm. (A) DW503, (B) BT604 and (C) ATCC 23344. The peaks around 16 ppm represent 6-deoxyhexose CH_3 signals while those around 21 ppm represent *O*-acetyl (CH_3CO) signals.

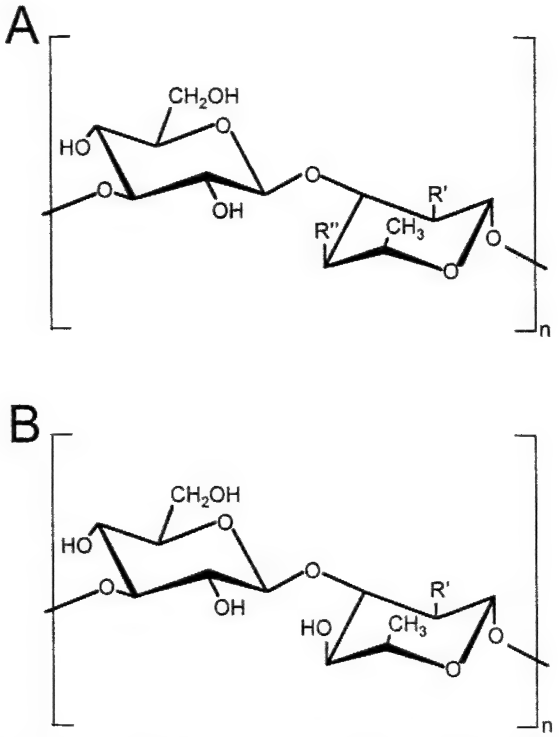

Fig. 5. Structures of (A) *B. pseudomallei* O-PS II and (B) *B. mallei* O-PS. In *B. pseudomallei* R′ = *O*-methyl or *O*-acetyl and R″ = *O*-acetyl or OH. In *B. mallei* R′ = *O*-methyl or *O*-acetyl.

3.4. Characterization of the epitope recognized by the Pp-PS-W mAb

We have recently demonstrated that the O-PS antigen expressed by *B. mallei* does not react with Pp-PS-W [11]. A comparison of the O-antigens expressed by *B. pseudomallei* and *B. thailandensis* with those expressed by *B. mallei* strains suggested that this phenomenon was likely due to differences in the *O*-acetylation patterns exhibited by the O-PS and O-PS II molecules (Fig. 5). Based upon the results of the current study, it is now apparent that the mAb reacts only with 3)-β-D-glucopyranose-(1→3)-6-deoxy-α-L-talopyranose-(1→ polymers in which the L-6dTal*p* residues are coordinately acetylated at the *O*-2 and *O*-4 positions. Whether or not the 2-*O*-acetyl modification imposes conformational constraints upon the O-polysaccharides or serves more directly as a structural epitope remains yet to be determined. Needless to say, however, these observations have proven to be a valuable re-

minder of the importance of maintaining the structural integrity of O-PS II during the synthesis of the glycoconjugate vaccine candidates.

Acknowledgements

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APPENDIX 4

Characterization of Class A β-Lactamase Mutations of *Burkholderia pseudomallei* That Confer Selective Resistance Against Ceftazidime or Clavulanic Acid Inhibition

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Running Title: *B. pseudomallei* β-Lactamase Mutations

Key Words: *Burkholderia*, β-lactamase, β-lactam resistance

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Abstract

Burkholderia pseudomallei, the causative agent of melioidosis, is inherently resistant to a variety of antibiotics including aminoglycosides, macrolides, polymyxins and β-lactam antibiotics. Despite resistance to many β-lactams, ceftazidime and β-lactamase inhibitor-β-lactam combinations are commonly used for treatment of meliodosis. Here, we examine the enzyme kinetics of β-lactamase isolated from mutants resistant to ceftazadime and clavulanic acid inhibition and describe specific mutations within conserved motifs of the β-lactamase enzyme which account for these resistance patterns. Sequence analysis of regions flanking the *B. pseudomallei penA* gene revealed a putative regulator gene located downstream of *penA*. We have cloned and sequenced the *penA* gene from *B. mallei* and found it to be identical to *penA* from *B. pseudomallei*.

Introduction

Burkholderia pseudomallei is the causative agent of melioidosis, an endemic disease of Southeast Asia and Northern Australia (7). The severity of the disease can vary from asymptomatic infection to a severe form leading to acute sepsis and death. *B. pseudomallei* is a facultative intracellular pathogen which is able to survive inside phagocytic cells and thereby escape the host's humoral response. The disease can be reactivated after a very long remission (4,6,13). Currently, prolonged antibiotic treatment is advised to ensure complete eradication of the organism. Unfortunately, this practice creates a strong positive selection for antibiotic resistant strains resulting in many cases of treatment failure. Many reports have described successful treatment using a combination of β-lactam antibiotics and a β-lactamase inhibitor, such as amoxicillin plus clavulanic acid (19). Livermore, *et al.* described a clavulanic acid inhibitable β-lactam resistant phenotype of *B. pseudomallei* (14), and recently, the cloning of *B. pseudomallei* class A and D β-lactamases has been reported (5,15).

Godfrey *et al.* described three different phenotypes of clinical isolates from three patients which had undergone antibiotic treatment, and demonstrated that the resistance was due to derepressed β-lactamase production and structural mutations in the enzyme (11). Here, we examine the *B. pseudomallei penA* gene encoding a class A β-lactamase in the clinical isolates of *B. pseudomallei* described by Godfrey, *et al.* and from *B. mallei* ATCC23344. We have identified point mutations in two of the isolates which likely account for their altered phenotypes. Finally, the enzyme kinetics of these mutants were compared to the wild type enzyme.

Materials and Methods

Bacterial strains and Plasmids. The bacterial strains and plasmid used in this study are shown in Table 1. *B. pseudomallei* strains used in this study were collected from blood and urine samples from melioidosis patients both before and during antibiotic treatment at Sappasitprasong Hospital, Ubon Ratchatani, Thailand, between 1986 and 1989 and have been described previously (8,11) . All bacterial strains were grown at 37° C on Luria-Bertani (LB) agar or in LB broth. Media used for growing *B. mallei* were supplemented with 4% glycerol. When used, antibiotics were added at the indicated concentrations.

PCR amplification and cloning of PCR products. PCR products were generated in a 100 µl reaction using the following cycling program: 95° C, 5 min; 95° C 1 min, 55° C 1 min, 72° C 1 min for 30 cycles; 72° C 10 min; then held at 4° C. Primers used in the amplification of the 580 bp *penA* from *B. pseudomallei* 1026b were 1) 5′-GCAGCACATCCAAGATGATG C-3' 2) 5'-GCCGATCGTGTTCATCGTCTA-3'. The primers used in reverse PCR to amplify flanking regions of *penA* using *Xho*I digested and relegated chromosomal DNA of 1026b were: 1) 5′out: 5′-GCATCATCTTGGATGTGCTGC-3′ 2) 3′out: 5′-GCCGATCGTGTTCATCGTCTA-3′. The primers used to amplify the entire *penA* gene were: 1) 5'penA: 5'-GAGAGCTGATACGCTAGCGAG-3' 2) 3'penA: 5'-GCGGCTTCCGGAAGGTTCA-3'. The zeocin resistance gene was amplified with 1) Zeo1: 5'-TGGCCTTTTGCTCACATGTGT-3' 2) Zeo2: 5'-TCTAGAGTCGACCTGCAGGCA-3'.

Cloning of β-lactamase genes from *B. pseudomallei* and *B. mallei*.

The *penA* gene was amplified from various *B. pseudomallei* mutants and from *B. mallei* using the 5' penA and the 3' penA primers. The PCR products were subsequently cloned into pCR2.1-TOPO (Invitrogen) as per the manufacturer's instructions. The cloned *penA* genes were

transferred from the pCR2.1-TOPO cloning vector to pUCP31T (18) for MIC testing and to pT7Zeo (Invitrogen) for β-lactamase expression. Restriction enzymes and T4 ligase were purchased from BRL/Invitrogen. Plasmids were purified using a QIAprep Spin Miniprep Kit (Qiagen), and chromosomal DNA was prepared using a Wizard DNA Purification Kit (Promega). When required, PCR products were purified using GenElute PCR DNA purification kit (Sigma).

MIC determination. MICs were determined using agar dilution or E-test strips (AB Biodisk, Solna, Sweden). For agar dilution, Mueller-Hinton agar plates were prepared containing 2-fold dilutions of antibiotic ranging from 1-256 μg/ml for ampicillin, amoxicillin, cefazolin, and from 0.25-128 μg/ml for amoxicillin plus clavulanic acid (8:1 ratio), cefoxitin, ceftriaxone and aztreonam, and from 0.25-128 μg/ml for ceftazidime and imipenem. Plates were spotted with approximately 10^4 organisms diluted from overnight liquid cultures and examined after overnight incubation. E-test strips were used as per manufacturer's instruction.

DNA sequencing and sequence analysis. DNA sequencing was performed by University Core DNA Services (University of Calgary). The CLUSTAL W program (21) was used to align *penA* genes and their translated protein sequences.

Purification of β-lactamase and analysis of enzyme kinetics.

The β-lactamase enzyme from *B. pseudomallei* 316a, 316c and 392f was purified in the following manner. *E. coli* BL21(DE3)(Invitrogen) cells were transformed with p316aT7Z or p392fT7Z, and from the transformants periplasmic proteins were obtained using an osmotic shock procedure.

For *B. pseudomallei* 316c β-lactamase purification, *E. coli* BL21(DE3)LysS (Invitrogen) was used in an effort to obtain higher β-lactamase expression. For osmotic shock, four liters of each *E. coli* transformant was grown over night, and cells were harvested by centrifugation and resuspended in 30 – 50 ml of 0.5 M sucrose for approximately 15 minutes. Periplasmic proteins were released by gently resuspending centrifuged cells in 20 ml sterile distilled water. The

periplasmic protein extracts were filter-sterilized and adjusted to 40 mM Tris-HCl, pH 8.5 to a final volume of 10 ml. The adjusted extracts were loaded into a Q-sepharose fast flow 16/20 chromatography column. The *B. pseudomallei* β-lactamase enzyme was collected in the pass through fraction. The pass through fraction was then concentrated to 2 ml with a Centriprep Centricon-10 and loaded into a MonoS HR 5/5 FPLC column (Amersham Pharmacia). The β-lactamase fraction was then eluted with a 0 to 2 M NaCl gradient to obtain a pure fraction of the enzyme. The pH of the enzyme extract was adjusted to 7.0, and this material was used for kinetic studies.

Enzyme purity was assessed by 14 % SDS-PAGE (data not shown). The kinetic analysis of β-lactam hydrolysis was performed with a Beckman DU640 spectrophotometer using 0.1 M phosphate buffer, pH 7.0.

Competition assays were performed in a total volume of 500 μl buffer in 5 or 10 mm quartz cuvettes. Reporter (nitrocefin) was added to a final concentration of 100 μM and inhibitor to a concentration of 50 or 100 μM. The extinction coefficients (Δε) and UV absorption wave length of each antibiotic used in this study were as follows: nitrocefin, +15,000 $M^{-1}cm^{-1}$ and 482 nm; ampicillin -1,100 $M^{-1}cm^{-1}$ and 232 nm; amoxicillin, -1,100 $M^{-1}cm^{-1}$ and 232 nm; cefazolin, -7,900 $M^{-1}cm^{-1}$ and 260 nm; cefoxitin, -7,700 $M^{-1}cm^{-1}$ and 260 nm; ceftriaxone, -9,400 $M^{-1}cm^{-1}$ and 260 nm; ceftazidime, -8,660 $M^{-1}cm^{-1}$ and 260nm; aztreonam, -640 $M^{-1}cm^{-1}$ and 318 nm; and imipenem, -9000 $M^{-1}cm^{-1}$ and 300 nm.

Km and *Vmax* were calculated using non-linear regression analysis by Prism software. The *kcat* was obtained using the known amount of enzyme measured by BCA protein assay (Pierce, Rockford, IL.). *Ki* was obtained using the method described by Galleni *et al.* (10) and was used as *Km* when the hydrolysis rate could not be measured.

Nucleotide sequence accession numbers. The *penA* sequences were submitted to GenBank under accession numbers AY032868, AY032869, AY032870, AY032871, AY032872, AY032873 and AY032874.

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Results

Reverse PCR and flanking region of the *penA* gene. Reverse PCR was performed in order to obtain the sequence of flanking regions of the *penA* gene. The orientation of *penA* and flanking genes are shown in figure 1. The *penA* gene is downstream of the *nlpD* gene which is presumably involved in lipoprotein synthesis and is upstream of a putative regulator gene, *penR*. The *nlpD*, *penA* and *penR* genes have the same orientation. There is approximately 150 bp and about 700 bp of intergenic regions between *nlpD* and *penA*, and *penA* and *penR*, respectively.

DNA sequence analysis. The PCR amplified *penA* gene from 6 different isolates of *B. pseudomallei* and *B. mallei* ATCC23344 was sequenced and compared using the CLUSTAL W program. The DNA sequences of these seven strains were almost identical in that only a few single base changes were identified. The presumptive translated protein sequences were identical between *B. mallei* and *B. pseudomallei* strains 316a (wild type phenotype), 365a (derepressed) and 365c (wild type phenotype). When strain 316c (ceftazidine resistant) was compared to strain 316a (wild type phenotype), a single nucleotide change (C to T) was found resulting in a change of proline to serine at position 167 (P167S, ABL numbering scheme (1)). The clavulanic acid resistant strain 392f had a single nucleotide change (C to T) at S72F. Nucleotide changes in both 316c and 392f resulted in amino acid changes within conserved regions of class A ß-lactamases. A single base change (T to C) in strain 392a (wild type phenotype) resulted in a substitution of methionine with threonine (M266T) and was outside of the conserved regions of class A ß-lactamases. Sequence comparison of the putative regulator region in all of the strains examined did not reveal any differences at the amino acid level (data not shown).

MIC determination in parental strains and their corresponding clones. The MICs of ten different β-lactam antibiotics were determined and compared in pairs of *B. pseudomallei* and *E. coli* TOP10 containing the corresponding *penA* clone. The results are shown in Table 2. Both

316c and TOP10 (p316c31T) had a relatively high MIC for ceftazidime, although TOP10 (p316c31T) was not significantly higher than many of the other *E. coli* Top10 *penA* clones. Also, both 392f and TOP10 (p392f31T) showed a small but consistent decrease in susceptibility to clavulanic acid inhibition, when compared with 392a or TOP10 (p392a31T).

β-lactamase purification and kinetic parameters. Enzyme obtained from periplasmic extracts and subjected to ion exchange column purification yielded β–lactamase with greater than 90% purity. The enzyme preparations were used to examine the kinetics of β–lactam hydrolosis in 3 of the *B. pseudomallei* strains. In general, the β-lactams used in this study could be divided into five groups. The first group consisted of "good" substrates, such as nitrocefin, cefazolin and ceftriaxone, which exhibited high k_{cat}/Km. The second group represented "poor" substrates, such as ampicillin and aztreonam. The third group were "very poor" substrates, such as amoxicillin, in that the hydrolysis rate could not be measured but could be derived by using the competitive hydrolysis method. The fourth group consisted of non-substrates as the enzyme could not recognize those β-lactams either as substrates or inhibitors. The last group was the inhibitor group and consisted of a single substrate, clavulanic acid. The hydrolysis rates of 9 different β-lactams, representing the 5 groups described above, were examined using the "good" substrate nitrocefin as a reporter and the *Ki* obtained from these competitive analysis experiments was used as a *Km* for comparison. The kinetic parameters obtained from β-lactamases from 3 of the *B. pseudomallei* strains are shown in Table 3. Ceftazidime was not recognizable by 316a and 392f enzymes; however, it was a substrate for the 316c enzyme, in that it was recognizable via competitive hydrolysis, albeit very poorly. Ampicillin and aztreonam were very poor substrates for the 316c enzyme yet good substrates for 316a and 392f. The *Km* (*Ki*) of 392f for clavulanic acid was about 5 fold higher than that of 316a indicating lower affinity of the 392f enzyme for clavulanic acid however, the 316c enzyme had the highest *Km* for clavulanic acid among the three strains.

Discussion

This study examines the *penA* gene and the class A β-lactamase enzyme which it encodes from several *B. pseudomallei* clinical isolates. In addition, we have sequenced the *penA* gene from *B. mallei* and have found it to be identical to that found in *B. pseudomallei.* We have shown that the β-lactamase resistant phenotype in *B. pseudomallei* can be attributed to amino acid changes in conserved regions of the β-lactamase enzyme.

Although the sequence of *B. pseudomallei penA* has recently been reported (5), reverse PCR experiments revealed a unique arrangement of the *penA* structural gene with a putative regulator downstream and in the same orientation. Sequence analysis of the putative regulator region did not reveal any differences between all *B. pseudomallei* strains examined at the amino acid level suggesting that the observed derepressed phenotypes are not a result of mutations within this region and that other factors contribute to the elevated enzyme levels in these strains.

The approximately 700 bp region which separates the *penA* gene and the putative regulator contains repeats and inverted repeats and may possibly contain unknown regulatory features. This region remains a target for further studies aimed at understanding the regulation of the β-lactamase enzyme.

The *penA* gene in *Burkholderia spp.* encodes a class A β-lactamase which is susceptible to clavulanic acid inhibition. The predicted protein sequence contains all four conserved motifs found in other class A enzymes, namely SXXK, SDN, omega loop (EXXLN), and KTG motifs (12), and according to its activity, *PenA* would be classified in the Bush group 2e (3, 5). The enzymes from two strains, 316c and 392f, had mutations that resulted in amino acid changes within the conserved motifs of the catalytic site. The mutation of 316c at the omega loop (P167S) may explain the observed ceftazidime resistance as this mutation has been shown to be associated with ceftazidine resistance in *K. pneumoniae* (16). Although the rates of ceftazidime hydrolysis by β-lactamase from 316c and 316a were not directly measurable, the 316c enzyme could recognize ceftazidime as competitive substrate and thereby allowed calculation of *Ki*, *kcat*,

and *kcat/Km* values. It is likely that the increased affinity for ceftazidime of the 316c enzyme may account for the increased resistance of this strain to this antibiotic.

The 392f enzyme also contained a point mutation in a conserved motif resulting in a S72F mutation. Although the phenylalanine at this position can be found in many β-lactamases, it is convincing that in this case, the change resulted in a decreased susceptibility to clavulanic acid inhibition in both *B. pseudomallei* strain 392f and in *E. coli* TOP10 (p392f31T) as the *Ki* for clavulanic acid was higher for the 392f enzyme than the wild type, 316a enzyme. The higher *Ki* would indicate decreased affinity of clavulanic acid, resulting in a higher MIC for amoxicillin plus clavulanic acid for strain 392f and *E. coli* TOP10 (p392f31T) as compared to strain 392a and TOP10 (p392a31T).

The *Ki* obtained for clavulanic acid and strain 316c was higher than for strains 392f and 316a, which may explain decreased susceptibility to clavulanic acid inhibition. However, the MIC of amoxicillin/clavulanic acid in 316c was not higher than 392f. This may be explained by the fact that 316c enzyme hydrolyzed amoxicillin more poorly than the wild type enzyme, so the decreased susceptibility to clavulanic acid inhibition could not raise the MIC of amoxicillin/clavulanic acid.

The MICs of *B. pseudomallei penA* genes cloned into *E. Coli* TOP10 may not accurately reflect actual enzyme activity in *B. pseudomallei*. Efflux mechanisms and/or differences in outer membrane permeability may alter periplasmic β-lactam concentrations and thus may affect apparent enzyme activity.

While this paper was being reviewed the cloning of a class D β-lactamase from *B. pseudomallei* has been reported (15). The authors reported increased transcription of the class D β-lactamase gene in laboratory generated ceftazidime resistant mutants. However, extracts of *E. coli* carrying the cloned gene from parent and mutant showed no detectable ceftazidime or imipenem hydrolying activity. Thus, the role of the *B. pseudomallei* class D β-lactamase in β-lactam resistance remains unclear..

Other factors outside the coding region of the *penA* gene may also contribute to highly resistant phenotypes as seen in 365a and 365c. Currently, we are studying the function of *penR*, the putative regulator and the intergenic 700bp region on the expression of β–lactamase in *B. pseudomallei*.

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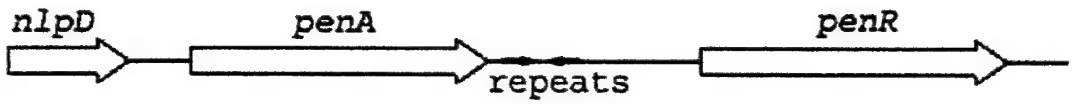
nlpD
penA
penR
repeats

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Reference or source
Strains		
E. coli TOP10	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)7697 *galU galK rpsL* (Str^R) *endA1* nupG	Invitrogen
BL21(DE3)	F- *ompT* $hsdS_B$ (r_B-m_B-) *gal dcm* (DE3) pLysS (Cam^R)	Invitrogen
BL21(DE3)pLysS	BL21(DE3)pLysS: F- ompT $hsdS_B$ (r_B-m_B-) gal dcm (DE3) pLysS (Cam^R)	Invitrogen
B. cepacia K56-2	CF Isolate, Toronto, Canada	8
B. mallei ATCC 23344	Type strain (human isolate)	USAMRIID*
B. pseudomallei 316a	Clinical isolate, wild type phenotype	10
B. pseudomallei 316c	Clinical isolate, selectively resistant to ceftazidime	10
B. pseudomallei 365c	Clinical isolate, wild type phenotype	10
B. pseudomallei 365a	Clinical isolate, derepressed phenotype	10
B. pseudomallei 392a	Clinical isolate, wild type phenotype	10
B. pseudomallei 392f	Clinical isolate, decreased susceptibility to clavulanic acid inhibition	10
B.thailandensis ATCC700388	Type strain (soil isolate)	2
Plasmids		
pCR2.1-TOPO	Topoisomerase-mediated cloning vector: Ap^r Km^r	Invitrogen
pEM7/Zeo	Expression vector, Zeo^r	Invitrogen

pUCP31T	Broad host range vector: OriT pRO1600 ori; Gm^r	18
p316a31T	pUCP31T containing cloned penA from 316a	This study
p316c31T	pUCP31T containing cloned penA from 316c	This study
p365a31T	pUCP31T containing cloned penA from 365a	This study
p365c31T	pUCP31T containing cloned penA from 365c	This study
p392a31T	pUCP31T containing cloned penA from 392a	This study
p392f31T	pUCP31T containing cloned penA from 392f	This study
pJES307	pT7-7 derivative, expression vector with T7 promoter	16
pT7Zeo	pJES307 derivative with disrupted bla_{TEM-1}, Zeo^r	This study
p316aT7Z	pT7Zeo containing cloned penA from 316a	This study
p316cT7Z	pT7Zeo containing cloned penA from 316c	This study
p392fT7Z	pT7Zeo containing cloned penA from 392f	This study

* United States Army Medical Research Institute of Infectious Diseases

Table 2. MICs (μg/ml) of different β-lactams for *E. coli*, *B. pseudomallei* and *B. mallei*.

Strain	Ampicillin	Amoxicillin	Amox/Clav.	Cefazolin	Ceftriaxone	Cefoxitin	Ceftazidime	Aztreonam	Imipenem
TOP10	4	4	4	2	<1	4	0.25	<1	0.25
TOP10 (p316a31T)	>256	>256	128	256	4	4	2	8	0.25
TOP10 (p316c31T)	256	>256	>128	>256	4	>128	4	2	1
TOP10 (p365a31T)	>256	>256	32	256	16	8	4	8	0.25
TOP10 (p365c31T)	256	>256	32	128	16	8	4	8	0.25
TOP10 (p392a31T)	128	>256	8	128	4	8	2	8	0.25
TOP10 (p392f31T)	128	>256	16	64	4	8	1	8	0.25
ATCC 23344	64	128	8	>256	8	>128	1	32	0.25
316a	64	128	8	>256	8	>128	4	32	0.5
316c	64	128	8	>256	8	>128	64	32	0.5
365a	>256	>256	128	>256	128	>128	16	>256	2
365c	64	128	128	>256	128	>128	16	>256	0.5
392a	64	128	16	>256	4	>128	2	32	0.5
392f	64	128	32	>256	8	>128	4	64	0.5

Table 3. Kinetic parameters of *B. pseudomallei* β-lactamases.

Substrate	*PenA*	*Vmax*	*Km*	*kcat*	*kcat/Km*	Substrate	*PenA*	*Vmax*	*Km*	*kcat*	*kcat/Km*
Nitrocefin	316a	159±7.4	10.9±2.1	4.91	446	ceftriaxone	316a	1514±389	287±117	63.1	219
	316c	67.6±4.2	3.34±0.7	1.01	303		316c	253±29.4	29.4±8.5	0.95	32.4
	392f	351±7.2	38±2.1	46.2	1214		392f	802±128	138±34.4	26.3	191
Ampicillin	316a	1316±114	126±29.5	0.18	1.45	ceftazidime	316a	NM	NR	NM	NM
	316c	NM	13.8*	0.04	2.74*		316c	NM	10.3*	0.02*	2.33*
	392f	4087±601	1051±237	37.2	35.4		392f	NM	NR	NM	NM
Amoxicillin	316a	NM	2.22*	NM	NM	aztreonam	316a	1645±211	179±61.4	3.2	17.9
	316c	NM	0.88*	0.09	96.9*		316c	NM	48.1*	0.15*	3.2*
	392f	NM	74.6*	NM	NM		392f	1939±144	336±51.9	17.6	52.5
cefazolin	316a	1024±106	59.5±17.8	21.35	359	Imipenem	316a	NH	NR	NH	NH
	316c	168±5.9	12.6±1.8	0.63	50		316c	NH	NM**	NH	NM**
	392f	2390±383	255±52.8	78.6	307		392f	NH	NR	NH	NH
cefoxitin	316a	NH	NR	NH	NH	Clavulanic acid	316a	ND	0.61*	ND	ND
	316c	NM	15.4*	0.02	1.02*		316c	ND	17.7*	ND	ND
	392f	NH	NR	NH	NH		392f	ND	3.18*	ND	ND

Units are $\mu M \cdot s^{-1}$ for *Vmax*, μM for *Km*, s^{-1} for *kcat*, and $mM^{-1} \cdot s^{-1}$ for *kcat/ Km*. NR, antibiotic was not recognized by that particular enzyme; NM, the rate of hydrolysis is too slow to be measured accurately; ND, not determined; NH, hydrolysis was not detected after 30 minutes. *, *Ki* was used as *Km*; **, the enzyme was completely inactivated by imipenem and no nitrocefin hydrolysis was detected.

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                  *       20        *       40        *       60        *       80        *      100
K96243     : MNHSPLRRSLLVAAISTPLIGACAPLRGQAKNVAAAERQLRELESTFDGRLGFVALDTATGARIAHRGDERFPFCSTSKMMLCAAVLARSAGEPALLQRR :
ATCC23344  : ..................................................................................................... :
316a       : ..................................................................................................... :
316c       : ..................................................................................................... :
365a       : ..................................................................................................... :
365c       : ..................................................................................................... :
392a       : ..................................................................................................... :
392f       : ..........................................................F.......................................... :
PPM-1      : ..................................................................................................... :

                  *      120        *      140        *      160        *      180        *      200
K96243     : IAYAKGDLIRYSPITEQHVGAGMSVAELCAATLQYSDNTAANLLIALLGGPQTVTAYARSIGDATFRLDRREPELNTALPGDERDTTTPAAMAASVHRLL :
ATCC23344  : ..................................................................................................... :
316a       : ..................................................................................................... :
316c       : ..........................................................S.......................................... :
365a       : ..................................................................................................... :
365c       : ..................................................................................................... :
392a       : ..................................................................................................... :
392f       : ..................................................................................................... :
PPM-1      : ..................................................A.................................................. :

                  *      220        *      240        *      260        *      280        *
K96243     : VGDALGAAQRAQLNAWMLGNKTGDARIRAGVPADWRVADKTGTGDYGTANDIGVAYPPNRAPIVFIVYTTMRNPNAQARDDVIASATRIAARAFA :
ATCC23344  : ..................................................................................................... :
316a       : ..................................................................................................... :
316c       : ..................................................................................................... :
365a       : ..................................................................................................... :
365c       : ..................................................................................................... :
392a       : ..........................................................T.......................................... :
392f       : ..................................................................................................... :
PPM-1      : ..................................................................................................... :
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Figure 1. Orientation of *penA* and *penR* in *B. pseudomallei* and *B. mallei*, *nlpD*, putative enzyme involved in lipoprotein synthesis; *penA*, class A β-lactamase gene; repeats, inverted repeats (not to scale); *penR*, putative regulator gene.

Figure 2. CLUSTAL W alignment of 9 β-lactamases from *B. pseudomallei* and *B. mallei*. K96243, *B. pseudomallei* K96243; ATCC23344, *B. mallei* ATCC23344; PPM-1, *B. pseudomallei* Hong Kong strain (5). Vertical dots indicate identical amino acids. Letters indicate changed amino acids. Amino acids are numbered in accordance with the Ambler (ABL) numbering scheme (1).